UNIVERSITY OF ALBERTA

DEVELOPMENT OF HEPATITIS B VIRUS AS VECTORS FOR GENE THERAPY

by

SUMONTA CHAISOMCHIT

A thesis submitted to the Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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September 30, 1998

"According to the seed that's sown

So is the fruit ye reap therefrom.

Sown is the seed and planted well.

Thou shalt enjoy the fruit thereof."

Samyutta, i.227

UNIVERSITY OF ALBERTA

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **DEVELOPMENT OF HEPATITIS B VIRUS AS VECTORS FOR GENE THERAPY** submitted by **SUMONTA CHAISOMCHIT** in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** in **VIROLOGY**.

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ABSTRACT

Hepatitis B virus (HBV) is attractive for application as gene delivery vectors for gene therapy of liver diseases because of its liver specificity. In this study, the feasibility of using HBV as replicative and nonreplicative viral vectors for liver gene transfer has been investigated.

An HBV replicative vector was developed by insertion of a foreign gene, the *tat* gene from the human immunodeficiency virus type 1 (HIV-1), into the tether region in-frame with polymerase gene (P) of the HBV genome. Expression of a functional *tat* protein (Tat) by this HBV construct was demonstrated by its transactivation activity on the HIV-1 long terminal repeat. Expression of Tat in chicken hepatoma and human cervical carcinoma cells was significantly lower than that expressed in human hepatoblastoma cells, thus indicating the cell type and species specificity of HBV. Expression of the functional Tat by this HBV replicative vector seemed to be regulated by the preS1 promoter and/or the C promoter. Replication competence of this HBV replicative vector was retained but at a reduced level of about 1.5% of the HBV wild type capability. Complete viral particles was produced from this vector.

Application of this HBV replicative vector for gene transfer appears to be limited by the size and the functional conformation of a foreign gene to be expressed. The Zeocin™ resistant gene (Zeo^R), a reporter gene about 100 base pairs larger than the *tat* gene, could not be expressed by an HBV replicative vector, possibly due to the inability of the Zeo^R protein to function in a fusion form.

An HBV nonreplicative vector was developed by insertion of the Zeo^R gene with a stop codon into the HBV genome using the same strategy used for construction of the HBV replicative vector. Functional Zeo^R protein was expressed by this vector. Replication of this HBV nonreplicative vector could be supported by *trans*-complementation with the polymerase protein.

The chicken anemia virus VP3 gene (CAV-VP3) or apoptin, which has potential use in cancer gene therapy, was expressed by both HBV replicative and nonreplicative vectors as detected by its apoptotic inducibility in human hepatoblastoma cells. However, the activity of the CAV-VP3 protein expressed from the HBV replicative vector seemed to be lower than that expressed from the HBV nonreplicative construct.

The infectivity of HBV recombinant particles produced from HBV vectors was also investigated using the *in vitro* infection assay presented by Lu and colleagues (*J Virol* 1996; **70**: 2277-2285). Study on the infectivity of HBVtat recombinant particles indicated that the HBVtat vector particles are infectious.

To my parents, my first teachers

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I may be able to summarize my academic experience in this thesis; however, it cannot truly reflect what I have learned and experienced throughout these years. I understand now why this degree is called Doctor of "Philosophy".

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ABBREVIATIONS AND DEFINITIONS

$A_{260:280}$	ratio of absorbance at 260 nm and 280 nm
ATCC	American Type Culture Collection
bp	base pair
Bq	becquerel; decay per second
$CaCl_2$	calcium chloride
CO_2	carbon dioxide
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
ddATP	2',3'-deoxyadenosine-5'-triphosphate
ddCTP	2',3'-deoxycytidine-5'-triphosphate
ddGTP	2',3'-deoxyguanosine-5'-triphosphate
ddTTP	2',3'-deoxythymidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DHBV	duck hepatitis B virus
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	ethylenediamino-tetraacetic acid
g (centrifugation)	standard acceleration of gravity: 9807 mm/s^2
G (prefix)	giga: 10^9
g (weight)	gram
GSHV	ground squirrel hepatitis virus
HBV	hepatitis B virus

HCl	hydrochloric acid
HHBV	heron hepatitis B virus
hr	hour
IgG	immunoglobulin G
k (prefix)	kilo: 10^3
kb	kilobase: 10^3 bp
kD	kilodalton
l	liter
m	meter
M	molarity; number of moles per liter of solution
MEM	minimum essential medium
$MgCl_2$	magnesium chloride
min	minute
mol	mole
mRNA	messenger RNA
n (prefix)	nano: 10^{-9}
NaCl	sodium chloride
NaOH	sodium hydroxide
NMWL	nominal molecular weight limit
p (prefix)	pico: 10^{-12}
PCR	polymerase chain reaction
Pfu	DNA polymerase from *Pyrococcus furiosus*
RNase	ribonuclease
SCID	severe combined immunodeficiency
sec	second
v/v	volume by volume
w/v	weight by volume

w/w	weight by weight
WHV	woodchuck hepatitis virus
μ (prefix)	micro: 10^{-6}
°C	degree celsius (centrigrade)

CHAPTER 1

Introduction

I. Hepatitis B virus

Of several viral causes of acute and chronic liver infections in humans, hepatitis B virus (HBV) is one of the most common, with more than 300 million chronically infected individuals approximately worldwide.[1] Since the identification of Australian antigen or hepatitis B surface antigen[2] and the identification of HBV infectious particles,[3] rapid progress has been made on the biological characterization of HBV and elucidation of its replication mechanism, even at the molecular level.[4-7] Advanced understanding of the natural specificity, transmission, and pathogenicity of HBV infection has led to success in development of vaccines to prevent HBV infection[8-10] and, possibly, of effective treatment for chronic hepatitis B disease.[11,12] Moreover, with the advent of gene therapy, which has made it possible to correct a disease phenotype by introducing new genetic material into affected cells using an organ-targeting vector, the increased understanding of HBV replication could lead to the development of HBV as a liver-specific gene delivery vector for gene therapy of liver diseases.

1.1 Hepatitis B Virus Infection

1.1.1 Route of transmission

Parenteral and percutaneous exposure of virus-containing blood or body fluids is the major route of HBV infection. Transmission can occur both vertically and horizontally. Maternal-neonatal vertical transmission represents a principle mode of hepatitis B

transmission in highly endemic areas, such as, China, Southeast Asia, sub-Saharan Africa and Pacific islands.[13] Horizontal transmission by parenteral and sexual contacts is the main route of hepatitis B infection in North America and Europe.[14,15] The rate of infection is highest in sexually active individuals.[14,15]

1.1.2 Clinical consequences

Primary HBV infection may be asymptomatic or may result in acute or fulminant hepatitis.[16,17] Most of the acute infections are self-limiting. About 90-98% of the patients recover completely and clear the virus, probably due to the function of cytotoxic T-lymphocytes (CTL) and antibody neutralization of extracellular particles.[18,19] Approximately 5-10% of infected adults develop chronic HBV infections which may be asymptomatic or exhibit varying degrees of severity of liver injury.[18,20] Although persistence of HBV infection is a minority outcome, it is of great importance because chronic HBV carriers represent the principle reservoir of the virus. Additionally, chronic rather than acute infections account for most of the mortality and mobidity of HBV infection.[20] Long term hepatitis B carriers seem to have markedly increased risks of developing hepatocellullar carcinoma (HCC).[21,22]

Clinical and experimental evidence suggests that variations in host immune response between individuals are responsible for differences in the degree of severity of infection and their ability to clear the virus.[23-25] Development of chronic hepatitis B may result from deficiency in the HBV-specific immune response, particularly cell-mediated immunity which is believed to play a pivotal role in clearance of HBV.[25-27]

To our knowledge, HBV is not directly cytopathic to hepatocytes. The association of HBV infection with liver cell injury or development of HCC is speculated to result from an excessive immune response against viral antigens expressed by infected hepatocytes.[25,28]

1.2 Molecular Biology of Hepatitis B Virus

1.2.1 Hepadnavirus family

HBV belongs to a family of related DNA viruses known as hepadnaviruses (for hepatotropic DNA viruses). Other members of this family include woodchuck hepatitis virus (WHV),[29] ground squirrel hepatitis virus (GSHV),[30] duck hepatitis B virus (DHBV)[31] and heron hepatitis B virus (HHBV).[32] All of these viruses have common properties of pronounced host specificity, liver tropism and a distinct replication mechanism. However, some characteristics of the avian viruses differ from those of the mammalian viruses. For instance, the avian viruses encode only two types of envelope proteins (large and small surface antigens) and lack the X gene coding sequence.[7] While chronic infection with the mammalian viruses causes diverse clinical manifestations and seems to be associated with eventual development of HCC, no pathogenic sequelae are apparently linked to the avian virus infection.

The restricted host range of HBV and lack of laboratory animal models have hampered *in vivo* studies of HBV replication. Efficient *in vitro* infection is limited to primary hepatocytes of appropriate host species. In addition, *in vitro* infection of human primary hepatocytes with HBV is not reliable since variations in the infection efficiency have been observed.[33] Current knowledge of the HBV life cycle is, therefore, based on *in vitro* transfection assays of HBV DNA into suitable liver cell lines and *in vitro* and *in vivo* studies of related hepadnaviruses, especially DHBV and WHV. However, the recent development of an HBV-transgenic mouse system has fulfilled part of the dream of having an HBV *in vivo* model and its use, no doubt, will provide further insights into HBV replication.[34,35]

1.2.2 Host range and tissue tropism

The unique biological features of HBV infection are species specificity and restricted hepatotropism.[36] Productive infections of HBV have been established only in humans, chimpanzees and certain species of higher primates including gibbons, rhesus monkeys, woolly monkeys and African Green monkeys.[6] The primary site of HBV replication is the liver[37,38] although HBV DNA, replicative intermediates and proteins have also been detected in nonhepatic tissues of hepatitis B patients, including peripheral blood mononuclear cells (PBMC), pancreas, bone marrow, kidney, bile duct epithelium, spleen and thymus.[19]

The factors responsible for highly restricted species and tissue tropism of HBV are not yet well defined but presumably are at the level of recognition of the specific cellular receptor by the viral envelope proteins.[39,40] The restriction of HBV replication to hepatocytes may be influenced by the liver specificity of the HBV promoters, *i.e.* requirement of liver-specific transcription factors for HBV gene expression. Studies on tissue specificity of the HBV promoter/enhancer elements has indicated that the stringent liver specificity of HBV may be regulated by the C and preS1 promoter (see Transcription).[6,41]

1.2.3 Viral particle

Electron microscopy of serum-derived preparations of HBV reveals three types of viral particles: (1) 42-nm double-shelled spherical particles known as "Dane particles", named after the discoverer,[3] (2) 22-nm spherical particles and (3) 22-nm filamentous particles of variable length (50-1000 nm).[6,7] These particles carry common antigens on their surfaces, collectively called hepatitis B surface antigen (HBsAg).[7] The Dane particles are mature, infectious virions comprised of an icosahedrally symmetric nucleocapsid of 30 or 34 nm surrounded by a detergent sensitive envelope (Figure 1.1).[7,42] The HBV outer envelope

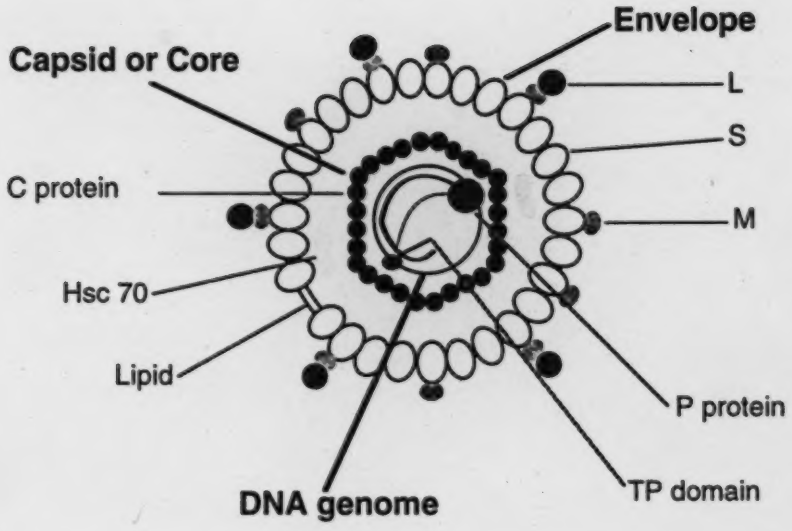


Figure 1.1 Schematic representation of the complete HBV virion structure (42 nm Dane particle). The outer envelope consists of the L, M and S or major surface protein subunits, embedded in a host-derived lipid bilayer. The icosahedral nucleocapsid contains the circular partially double-stranded DNA genome linked to the viral DNA polymerase protein (P) via the terminal protein domain (TP). A cellular heatshock protein Hsc70 has been reported to coexist in HBV virions. (Adapted from Nassal, 1997)[12]

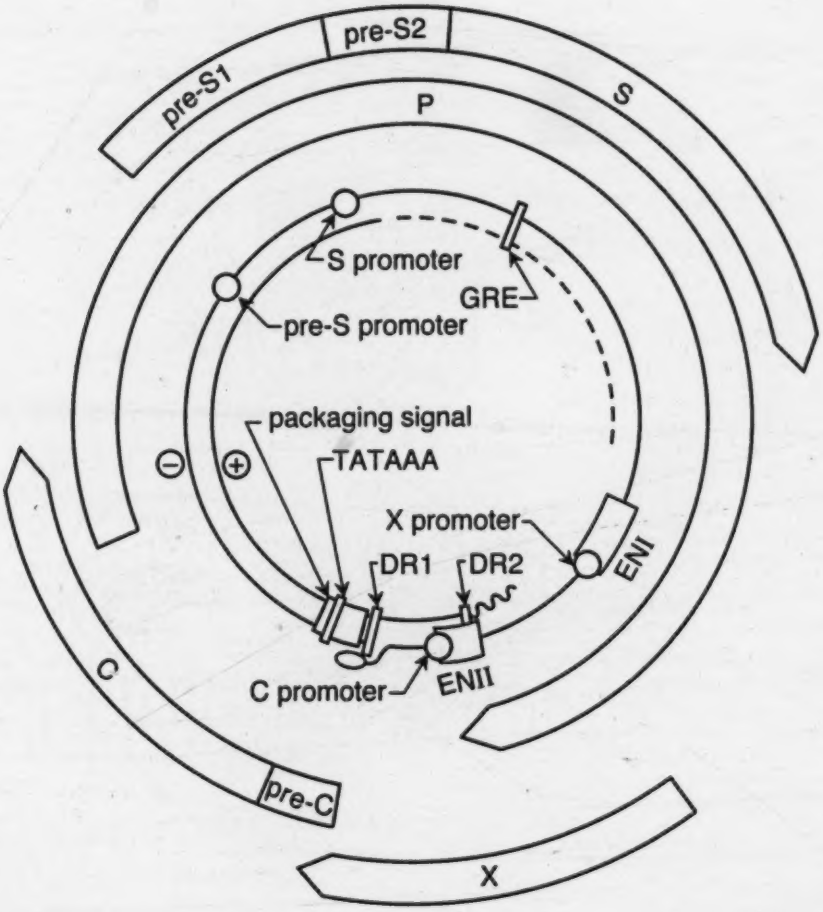
consists of the large (L), middle (M) and small or major (S) surface proteins in a lipid membrane.[43] The ratio of the quantity of L:M:S: subunits is approximately 1:1:4.[44] The viral nucleocapsid formed by identical subunits of capsid or core protein contains a circular partially double-stranded DNA genome of approximately 3.2 kb and a virus-encoded DNA polymerase covalently attached to the minus strand DNA.[42,45] A cellular heatshock protein Hsc70 has been reported to coexist in the virion.[12]

The spherical and filamentous particles, which are about 1000 times more abundant than the Dane particles, are composed solely of the HBV surface proteins and host-derived lipid.[6,7] These particles contain predominantly the S protein with variable and trace quantities of the M and L subunits, respectively. Since they are devoid of viral genetic material, these particles are noninfectious. Paradoxically, these subviral particles are strongly immunogenic.[46,47] The role of these particles in the HBV life cycle is not clearly understood. It has been speculated that they may act as decoys for the host immune system, thus facilitating the progression of infection by complete virions. Recent studies on a DHBV model have also suggested that these subviral particles may enhance viral replication and gene expression, possible through a mechanism triggered by binding of the L protein component to the cellular receptor.[39,48]

1.2.4 Viral genome

The HBV genome is a relaxed circular, partially double-stranded DNA of approximately 3.2 kb, one of the smallest viral DNA genomes known.[4,5] This genomic molecule possesses the unusual structure of an asymmetric DNA duplex whose circularity is maintained by a 5' cohesive overlap of the minus and plus strand DNA (Figure 1.2). The minus strand DNA is a complete unit length with its 5' end covalently linked to the viral polymerase via the terminal protein domain.[49] The plus strand DNA is incomplete and variable in length with a heterogeneous 3' end terminated 30-50% short of its completion.[50]

Figure 1.2 Genomic organization of HBV. Arrows represent the open reading frames preC/C, P, preS1/preS2/S and X. A solid (-) line represents the fully synthesized minus strand DNA. The incomplete plus strand DNA is indicated by a solid, hatched (+) line. Attached to the 5' ends of the minus and plus strand DNA are the terminal protein domain of the viral polymerase (an oval) and an RNA primer (a wavy line), respectively. The four promoter regions identified on the HBV genome are indicated as circles on the map and labeled accordingly. Other important regulatory elements are indicated, including direct repeat 1 (DR1), direct repeat 2 (DR2), enhancer I (ENI), enhancer II (ENII), polyadenylation signal (TATAAA), packaging signal and glucocortocoid-responsive element (GRE). (Modified from Lau and Wright, 1993[1] and Nassal and Schaller, 1993)[5]


pre-S2
pre-S1
P
S
S promoter
GRE
pre-S promoter
packaging signal
TATAAA
X promoter
DR1
DR2
ENI
ENII
C promoter
C
pre-C
X

The 5' end of the plus strand DNA bears a capped oligoribonucleotide.[51] The 11-nucleotide direct repeats, DR1 and DR2, which are important for initiation of synthesis of the plus and minus strand DNA, respectively, are located at the 5' end of the respective strands.

The HBV genomic structure is extremely compact, *i.e.* almost all nucleotides are included in coding sequences and more than half of the total sequence is used for more than one open reading frame (ORF).[5] All regulatory elements also overlap with coding sequences. Four major ORFs are encoded on the genome. The envelope or preS1/preS2/S ORF codes for the expression of the L, M and S surface proteins which are delineated by three in-frame initiation codons. The preC/C gene contains two in-frame initiation codons for the expression of hepatitis B e antigen (HBeAg) and capsid or core protein, respectively. The largest ORF, polymerase gene (P), codes for the viral polymerase protein (Pol) which is essential for genomic DNA replication. It overlaps with the C terminus of the C gene, the entire envelope ORF and the N terminus of the X gene which is carried by the smallest ORF.

Four promoters have been defined on the HBV genome, including the C promoter regulating transcription of heterogeneous genomic transcripts, preC mRNA and pregenomic RNA, the preS1 promoter for expression of preS1/preS2/S ORF, the preS2/S promoter for expression of heterogeneous preS2/S transcripts and the X promoter controlling transcription of the X ORF.[41] Recent studies have reported that transcription of preC messenger RNA (mRNA) and pregenomic RNA may be regulated by two distinct promoters overlapping on the basal C promoter.[52] Two enhancer elements, designated as enhancer I and enhancer II, have been identified upstream of the X promoter and the C promoter, respectively.[53,54] These enhancer elements have been shown to be involved in regulation of HBV gene expression in a liver-specific manner.[55-60] A glucocorticoid-responsive element (GRE)[61,62] and a retinoic acid responsive element (RARE),[57] which

have also been identified on the HBV genome, may exert their functions on tissue specificity in conjunction with the HBV enhancers.

1.2.5 Hepatitis B virus replication cycle

In vivo and in vitro studies of hepadnaviruses using molecular biological techniques has provided information leading to a schematic model for HBV replication (Figure 1.3). The HBV life cycle is initiated by attachment of the virion to a susceptible cell, presumably through a specific cellular receptor,[39,63] followed by endocytosis and release of the nucleocapsid by membrane fusion.[64,65] After cellular entry, the viral genome is imported to the nucleus with subsequent conversion of the relaxed circular, partially double-stranded DNA to covalently closed circular DNA (cccDNA). These steps are not well understood. However, it is known that cccDNA is the first virus-specific DNA species to appear, preceding the accumulation of viral RNA.[66] The cccDNA is transcribed by the host transcriptional machinery giving rise to a pool of genomic and subgenomic RNAs which are translated using host translational machinery into structural and functional viral proteins. One species of the genomic transcripts, termed pregenomic RNA, is selectively packaged together with the polymerase protein into viral capsids in the cytoplasm. Within these capsids, viral DNA replication occurs by synthesis of the minus strand DNA concomitantly with degradation of the RNA template followed by the plus strand DNA synthesis.[5] Newly formed nucleocapsids may re-enter the nucleus, resulting in amplification of the cccDNA pool, or may leave the host cell by budding into the ER to acquire the glycoprotein envelope.[67,68] Enveloped viruses are finally secreted via the constitutive pathway of cellular vesicular transport.[69]

Unlike retroviruses, host chromosomal integration is not an obligatory step in the basic HBV replication cycle, even though integration into the host DNA is often found in long term HBV-chronic carriers.[70,71]

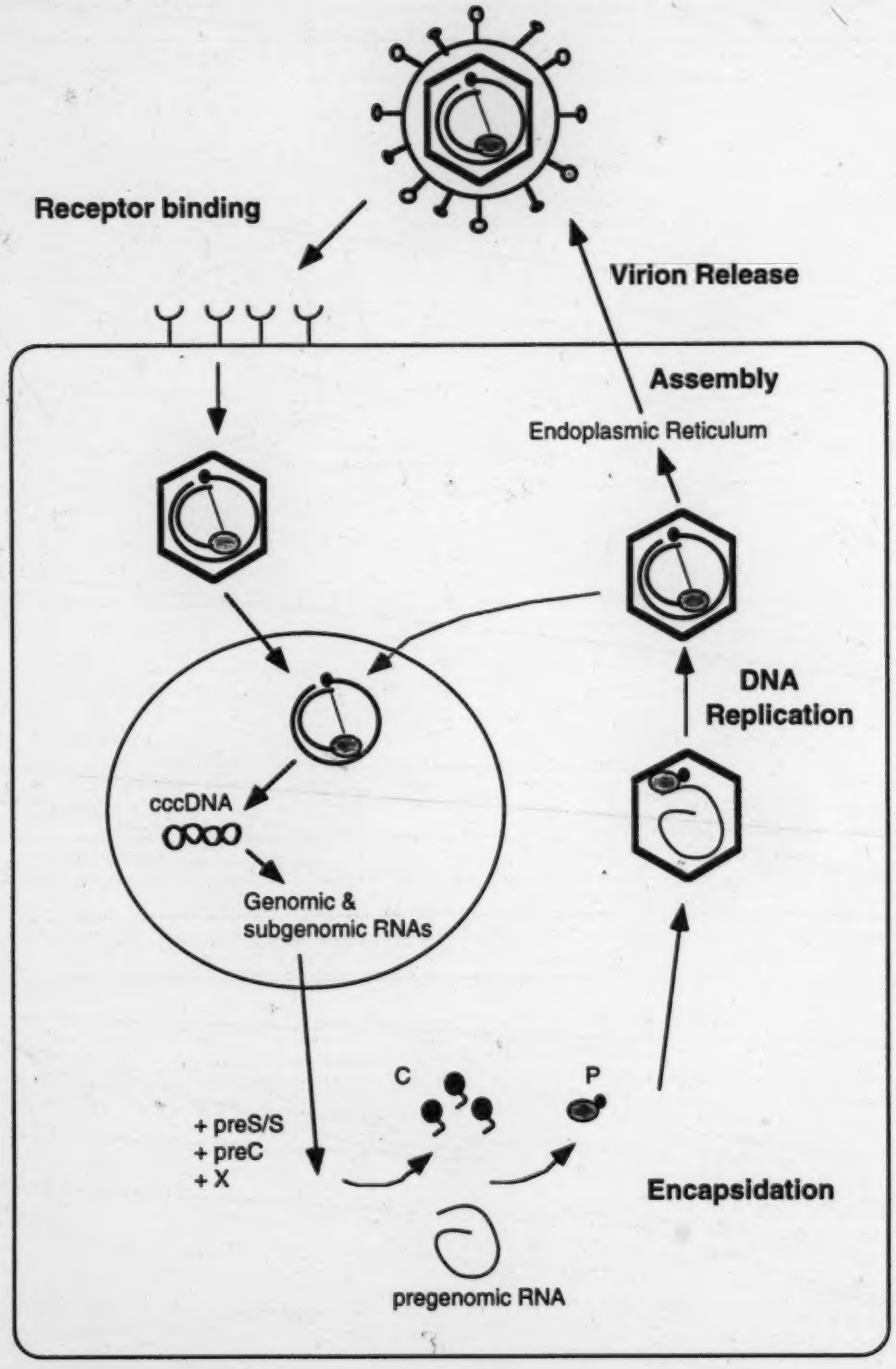


Figure 1.3 HBV replication cycle. (Adapted from Nassal, 1997)[12]

Attachment and cellular entry

The earliest events of HBV infection are the most poorly elucidated steps in the HBV life cycle. According to several lines of evidence, HBV enters the host cell by recognition of a specific cellular receptor(s) on the cell surface through its surface proteins. The preS1 domain of the L surface protein has been reported to play a pivotal role in receptor recognition.[72-75] Specifically, the 21-47 amino acid sequence of the preS1 domain has been demonstrated to encompass the epitope responsible for binding.[72,73,76,77] Antipeptide antibodies to this region block adherence of HBV virions to a human hepatoblastoma cell line (HepG2).[78,79] However, none of the studies has directly demonstrated its involvement in productive HBV infection. One strong but indirect inference of the importance of preS1 domain in viral infectivity comes from genetic studies of DHBV and HHBV host range determination.[40] Replacement of as few as 69 amino acids of the preS domain of the HHBV L protein with the DHBV counterpart sequences allows the chimerical HHBV to infect duck hepatocytes.

Circumstantial evidence suggests that the S surface protein also may be involved in cellular attachment and internalization. Radioactively labeled S protein has been demonstrated to bind specifically to intact human hepatocytes and liver plasma membrane.[80,81]

Much less is known about the cellular receptor responsible for ligand recognition in HBV infection. A variety of proteins derived from liver or serum have been identified for binding to the HBV surface proteins or their polypeptide derivatives.[75,76,81-83] Products of 35 and 50 kD molecular weight extracted from HepG2 cells have been shown to bind specifically to the recombinant HBV preS1 sequence and to block the attachment of preS1-positive HBV particles to HepG2 cells.[75,76] Hertogs *et. al.*[81] have reported the specific binding of a plasma membrane protein, Endonexin II, to the S protein. The identification of nonmembrane-bound proteins, for instance, polymerized human serum albumin,[84,85]

human apolipoprotein H[83] or a 50 kD serum-derived binding factor,[82] which are able to bind to the HBV surface proteins has raised the idea of an "intermediate carrier" for facilitating HBV cellular entry. Alternatively, these proteins might act as carriers in delivery of HBV particles from the periphery of the body to the liver. Other studies also indicate possible roles for other molecules, including IgA,[86] interleukin-6,[87] Vero cell receptor[88,89] and transferrin receptor,[90] for the cellular entry of HBV. However, none of these molecules has been convincingly associated with a productive HBV infection.

Upon binding, HBV is taken up into the host cell most likely by endocytosis.[64,65] Recent DHBV infection studies have shown that the uptake of the virus into primary hepatocytes is ATP-dependent[64] and is inhibited by lysosomotropic agents.[65] However, evidence for pH dependence of the uptake mechanism is not yet conclusive.[64,65,91]

Nuclear transport and formation of cccDNA

Since HBV transcription is performed by the host machinery present in the nucleus, nuclear localization of the viral genome must occur, but the actual mechanism involved in this process is still not well understood. For several reasons, however, it is highly unlikely that the complete nucleocapsid containing the viral genome is transported into the nucleus. The diameter of an HBV nucleocapsid, 30 or 34 nm,[42] exceeds the limit of the functional nuclear pore size, maximum diameter about 20 nm.[92] Moreover, studies have demonstrated that the nuclear membrane presents a impermeable barrier for the intact HBV nucleocapsid.[93,94] Therefore, the complete or partial disintegration of the nucleocapsid structure prior to the nuclear translocation of the viral genome may be assumed. Recent experimental studies have indicated that the disassembly possibly occurs at the nuclear membrane, resembling the dissociation of an adenoviral nucleocapsid.[94-96] The translocation of the viral DNA inside the nucleus may be mediated by the capsid or polymerase proteins which have been shown to contain nuclear localization signals (NLS).[97,98] Recent *in vitro* studies of WHV DNA nuclear transport have shown that the

polymerase-DNA complex extracted from complete WHV nucleocapsids is sufficient to be transported into the nucleus and the process appears to be ATP-dependent.[96] Infection studies in a DHBV system have further suggested the involvement of the cyclic-AMP (cAMP)-mediated regulatory pathway in DHBV DNA nuclear translocation, thus indicating the association of protein kinase A activity with the process.[99] These findings imply that changes in the phosphorylation state of the proteins involved, possibly capsid or polymerase, are necessary for nuclear importation of the viral genome.

Once in the nucleus, the circular partially double-stranded DNA is converted to cccDNA. The process involves: (1) removal of the 5' terminal structures, *i.e.* the oligoribonucleotide from the plus strand DNA and the polymerase protein from the minus strand DNA, (2) repair of the single-stranded gap on the plus strand DNA and (3) covalent ligation of both DNA strands. The possibility that these steps are performed by the cellular machinery is supported by experiments which showed that the formation of DHBV cccDNA is independent of the viral polymerase function.[100]

Transcription

HBV utilizes the host nuclear transcriptional machinery for transcription of its cccDNA genome. The process, however, is operated by viral promoters and enhancer elements.[7,41] Studies in HepG2 cells indicate strength of the HBV promoters in this order: C>X>preS2/S>preS1.[55] However, this order differs from the steady-state ratio of viral transcripts observed *in vivo* which indicates promoter strengths in the order of preS2/S>C>>preS1>>X.[38] The C and preS1 promoters display strong liver specificity.[41,101-103] The tissue specificity of these promoters may be influenced by the activation function of enhancer I and enhancer II through the binding of liver-enriched regulatory factors, such as hepatocyte-nuclear factors (HNF) and nuclear factor CCAAT/enhancer binding protein (C/EBP).[56,104-106] The preS2/S promoter also can be upregulated by the liver-specific enhancer II.[56,107] However, this promoter appears to be

constitutively active in a wide variety of cell types as shown by *in vitro* studies.[108,109] The X promoter also demonstrates little tissue specific activity.[55,101]

Two major classes of transcripts are produced; the 3.5 kb terminally redundant genomic RNAs and the 2.4 kb, 2.1 kb and 0.9 kb subsets of the subgenomic transcripts (Figure 1.4 and Table 1.1). All these transcripts are 5' capped, of plus-strand polarity, unspliced and polyadenylated at a single, common position within the C gene.[4] Although splicing does not seem to play an important role in the basic HBV life cycle, spliced derivatives of the HBV genomic RNAs have been observed in culture systems.[110-112] Mutational inactivation of the spliced sites, however, does not affect HBV replication in these cells.

The heterogeneous genomic RNAs are divided into two subsets of preC mRNA and C mRNA or pregenomic RNA, depending on the presence or absence of the preC translation initiation site.[4] Their transcription is controlled by the C promoter[41] or probably by two distinct regulatory elements overlapping on the basal C promoter.[52] These genomic transcripts comprise the entire genomic length with one redundant copy of the terminal direct repeat at the 3' end.[50] The preC mRNA serves as a template for synthesis of a preC protein for a secretory HBeAg whose function is still unknown. Translation of the preC ORF, however, interferes with pregenomic RNA packaging.[113] The pregenomic RNA is the most abundant and indispensable for viral genomic replication. It has dual functions, serving as a template for viral DNA replication and as an mRNA for translation of the C and P genes. The 5' end of the pregenomic RNA is unique and located about five nucleotides downstream of the preC initiation codon.[38]

The preS1 transcript of 2.4 kb, whose synthesis is operated by the preS1 promoter, contains all the preS1, preS2 and S regions of the envelope gene. It codes for translation of the L protein.

The subgenomic RNA family of 2.1 kb is another major RNA species produced. These transcripts also display heterogeneous 5' ends, bracketing the preS2 initiation codon.[4] The preS2/S transcript serves as a template for synthesis of the M protein subunits

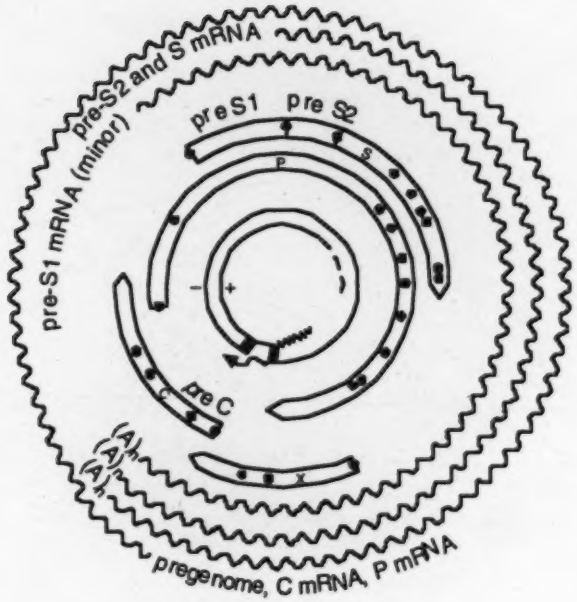


Figure 1.4 Diagrammatic representation of the genomic and subgenomic transcripts of HBV. Arrows represent the HBV open reading frames (ORFs). (+) and (-) lines represent the minus and plus strand DNA, respectively. The major viral genomic and subgenomic transcripts of HBV are indicated by wavy lines and labeled accordingly. The 3' end polyadenylated sequence of each transcript is indicated by (A)n. Black dots in the ORF representations indicate initiation and in-frame ATG codons. See text for more details. (Modified from Ganem and Varmus, 1987)[4]

Table 1.1 HBV gene products[a]

Gene	Protein product		mRNA	
	Name	Size (kD)[b]	Name	size (kb)
Envelope	Large (preS1/preS2/S)	43	preS1 mRNA	2.4
	Middle (preS1/preS2)	33	preS2/S mRNA	2.1
	Small (S)	24	S mRNA	2.1
preC/C	capsid (C)	21	pregenomic RNA	3.5
	HBeAg (preC)	18	preC mRNA	3.5
P	polymerase or Pol	94	pregenomic RNA	3.5
X	X	17	X mRNA	0.9

[a] Data are obtained from Schaller and Fischer, 1991;[41] Raney and MaLachlan, 1991[6] and Ganem, 1996.[7]

[b] Molecular weights indicated were estimated for unglycosylated proteins.[6]

of the HBV envelope whereas the other shorter species, the S transcript, is for the S protein translation. The synthesis of these RNA species is apparently regulated by the upstream preS2/S promoter.

The X transcript is weakly expressed in HBV-infected liver. Its expression was not detected until an *in vitro* transfection system became available. Transcription of this 0.9 kb RNA is controlled by the X promoter which displays strong activity in driving reporter gene expression *in vitro*.[55] The low level of the X gene expression by the X promoter *in vivo* is still unexplained.

Translation and viral proteins

HBV utilizes the host translational machinery for its protein synthesis. HBV encodes overlapping ORFs, which often contain multiple initiation codons to generate structurally similar but functionally different proteins, in order to maximize the limited capacity of its DNA genome. To ensure translation of the internal initiation codons, since internal initiation by the cellular translational system is inefficient,[114] the virus has a strategy of transcribing separate mRNA molecules beginning at the ATG codons of almost all ORFs, except the ATG of the P ORF. Each protein, except the Pol protein, is translated from its own individual mRNA (Table 1.1).

Surface or envelope proteins

The HBV envelope contains three species of surface protein: L, M and S (Table 1.1). These proteins differ in size and in N terminal sequences but share the C terminal S domain. They exist in both glycosylated and unglycosylated forms.[6,7] The S and M are the predominant components of HBV subviral particles, which contains only trace amount of the L polypeptide. HBV mature particles, however, are substantially enriched in the L subunit which seems to play a major role in viral assembly and infectivity.[7,115] The L protein seems to adopt two different transmembrane topologies due to posttranslational

modifications.[116] The L subunit with cytoplasmic preS1 domain seems to be involved in virion morphogenesis, probably via interaction with viral nucleocapsid.[116] The L protein containing the exposed N-terminal preS1 domain is probably responsible for recognition of a hepatocyte receptor and determination of species and liver specificity.[73,74,116] Myristylation of the L polypeptide may be involved in HBV adsorption and cellular entry since unmyristylated virions derived from mutations of the L protein are noninfectious.[117,118] The preS2 and S domains have also been reported to play a role in HBV infection.[74,119,120]

Hepatitis B e antigen

HBeAg is generated from a posttranslational modification of preC protein expressed from the preC mRNA. The preC protein contains a short N-terminal sequence, absent in the capsid polypeptide, which serves as a signal sequence to direct the preC protein to the secretory pathway.[121-123] The protein is processed in the endoplasmic reticulum (ER) and secreted through the vesicular transport system.[124,125] Cleavage of the signal sequence and the basic C-terminal region of the protein occurs during this process. HBeAg does not appear to be essential for HBV replication.[126,127] It, however, is thought to be involved in the induction of immunological tolerance.[128] The correlation between HBeAg expression and establishment of chronic infection in newborns from HBV-infected mothers has been demonstrated.[129]

Capsid or core protein

HBV capsid protein or hepatitis B c antigen (HBcAg) is synthesized from the first cistron, *i.e.* the C ORF, on the pregenomic RNA. This protein is a principle component of viral capsid and plays an important role in encapsidation of the pregenomic RNA (see RNA encapsidation). The basic arginine-rich C terminal domain is required for viral RNA packaging but not for capsid assembly[130,131] which is essentially mediated by the N

terminus.[130,132] Small deletions of the basic C terminus severely affect pregenomic RNA encapsidation and influence proper activity of the viral DNA polymerase.[130,132] Insertions within the first 131 amino acids of the capsid protein significantly decrease the efficiency of pregenomic RNA encapsidation as well as capsid formation.[133]

It has been shown that the capsid protein is phosphorylated *in vivo*.[134-136] However, studies in a DHBV model have suggested that phosphorylation of the capsid protein is not required for encapsidation of the pregenomic RNA.[137] It, however, has been shown to stimulate viral DNA replication in nucleocapsids and facilitate establishment of infection by mature virions.[137,138] These experimental data implicate that differential phosphorylation of the capsid protein may be necessary for a viral nucleocapsid to carry on its sequential facilitating functions in viral replication.

Polymerase protein

The HBV Pol protein is translated from the pregenomic transcript which is also used for the capsid protein synthesis.[5] The actual mechanism of Pol translation is still not clear but it is undoubtedly separated from the synthesis of the capsid protein.[139,140] Current data suggests that the mechanism may involve ribosomal leaky scanning or internal entry.[139,141,142]

The HBV Pol structure is divided into three major functional domains: the terminal protein domain (TP) at the N terminus, DNA polymerase/reverse transcriptase domain (Pol/RT) in the center and the RNase H domain at the C terminus. The TP and Pol/RT domains are linked by a nonessential spacer or tether region.[143,144] The TP is important for priming of the minus strand DNA synthesis while replication of the minus and plus strand DNA is exclusively dependent on the Pol/RT and RNase H activities (see Minus and Plus strand DNA synthesis). The Pol protein product is also necessary for encapsidation of the pregenomic RNA.[145,146]

X protein

The pleiotropic 17 kD protein product of the X mRNA is a promiscuous transactivator as it can transactivate not only the HBV promoters but also several other viral and cellular targets.[147-150] The transactivation function of the X protein is not yet well elucidated but it may involve protein-protein interaction since HBV X is not a DNA binding protein. The X protein has been shown to interact with several cellular transcription factors, such as AP1, AP2, TATA-binding protein and RBP5 subunit of RNA polymerase.[151-153] HBV X has been found in both the cytoplasm and nucleus of transfected cells, suggesting that it may interact with cellular proteins localized in both compartments.[154] It also has been reported to possess ribo-deoxy ATPase activity[155] and protein kinase activity[156] and to modulate cellular signal transduction pathways.[157,158]

Although the exact role of the X protein in HBV replication cycle is still unknown, this protein seems to be essential for HBV replication *in vivo*.[159,160] The expression of the X protein is thought to be involved in HBV-associated HCC. Studies in transgenic mice, however, provide contradictory results as some transgenic mice expressing high levels of the X gene product develop liver cancer[161] but other do not.[162,163] Nonetheless, HBV X seems to serve as a cofactor for HCC development in transgenic mice which do not develop spontaneous tumors.[162,164] HBV X has also been shown to interact with tumor suppresser p53 and interfere with its functions.[165-167] It also can upregulate some protooncogenes like *c-fos*, *c-jun* and *c-myc*.[149,168] Recently, a cellular X-associated protein-1 (XAP-1) which is thought to be involved in repairing damaged DNA has been identified.[169,170] Subcloning of the X domains which are important for binding to XAP-1 into HepG2 cells has been shown to inhibit the cellular repair mechanism.[171] Therefore, the possible mechanism of the HBV X association with HCC development in HBV chronic patients might be related to interference with the cellular DNA repair mechanism.

Pregenomic RNA encapsidation

The basic strategy of HBV replication involves reverse transcription of the pregenomic RNA intermediate which takes places within viral nucleocapsid.[172] Encapsidation of the HBV pregenomic RNA into viral capsid is initiated by direct interaction between the viral Pol protein and a *cis*-acting packaging element, known as the ε element, on the pregenomic RNA.[146,173,174] Packaging of the Pol protein is also dependent on its binding to the ε sequence.[173]

Because of the terminal redundancy of the pregenomic RNA, the ε element, encompassing approximately 100 nucleotides, is present at both ends of the RNA sequence. However, only the 5' copy located near the 5' DR1 region serves as an encapsidation signal for pregenomic RNA packaging.[175] Deletions of the 3' ε element of DHBV do not effect pregenomic RNA encapsidation, which may explain the exclusion of the subgenomic RNAs containing only the 3' ε sequence from encapsidation.[176] The virus also has a stringent mechanism for selective packaging of only the pregenomic RNA but not the preC mRNA which also bears the 5' ε element. This selectivity may involve ribosomal-mediated suppression associated with translation of the preC gene.[177]

Secondary structure analysis of the ε sequence reveals a characteristic stem-loop structure, containing a lower stem, a six nucleotide bulge, a upper stem with a single unpaired U residue and a six nucleotide loop (Figure 1.5). This latter is crucially important for the encapsidation mechanism.[7,178,179] Mutational studies of the requirements for encapsidation suggest that, in addition to the overall structure, the sequence of the loop and the first two of the six bulge nucleotides are critical for efficient interaction with the Pol protein.[179,180] Surprisingly, the sequence of the other four nucleotides of the bulge is not significantly important for pregenomic RNA encapsidation but appears to be essential for reverse transcription.[178,181] The requirements for HBV encapsidation are quite complex since mutations of the loop which severely impair the packaging do not abolish the Pol-ε

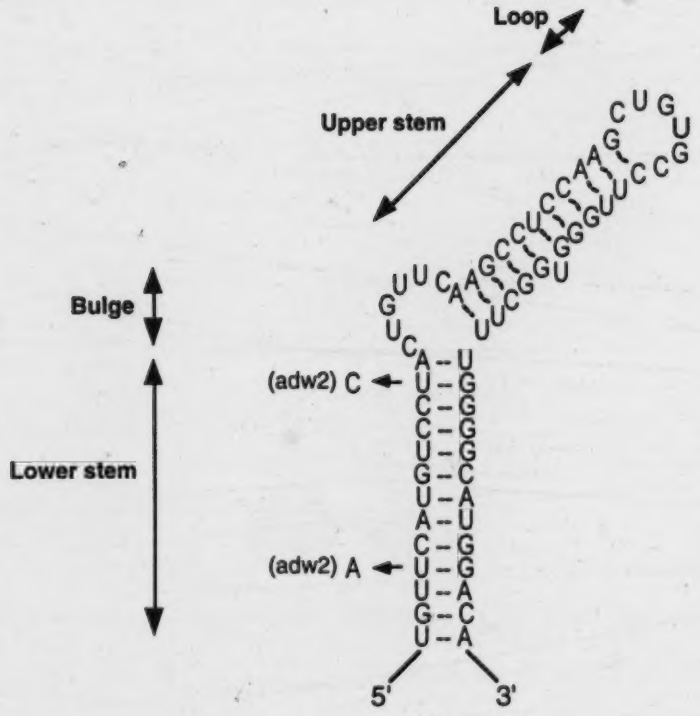


Figure 1.5 Computer analysis of RNA secondary structure of the ε sequence of HBV. This schematic figure represents the ε stem loop structure of HBV subtype ayw. Nucleotide changes in the adw subtype are also indicated by arrows. (Adapted from Ganem, 1996).[7]

binding.[182] Therefore, additional factors, possibly capsid proteins or host factors, may be required for efficient pregenomic RNA packaging. Some evidence suggests that heat shock protein Hsp90 also takes part in facilitating the interaction between Pol and pregenomic RNA.[183]

To complete the encapsidation process, the Pol-ε complex must become associated with the assembling capsid subunits. The details of this step are poorly understood but it may involve the interaction of the capsid structure with Pol or pregenomic RNA or both. The highly basic C-terminal region of capsid protein, which can function as a nucleic acid binding domain, has been shown to be required for pregenomic RNA encapsidation.[131] No direct evidence of the interaction between the Pol and capsid proteins have been demonstrated. However, the C-terminus of the Pol protein which is nonessential for the ε binding is still important for the packaging *in vivo*.[174,182] This has led to a model in which the major part of the N-terminus of Pol interacts with the pregenomic RNA and association of this complex with the assembling capsid structure is mediated by its C-terminal portion.

An interesting feature of the HBV encapsidation mechanism which should be noted is the distinctive preference of the Pol protein product to function in *cis*, *i.e.* preferentially to encapsidate its own pregenomic RNA.[174,184] One possible explanation is that the nascent Pol protein cotranslationally binds to its own mRNA.[174,184]

Minus strand DNA synthesis

The encapsidation of pregenomic RNA is correlated with initiation of genomic DNA replication.[185] Binding of the Pol protein to the 5' ε sequence not only triggers the viral nucleocapsid assembly but also activates reverse transcription for minus strand DNA synthesis.[185,186] The ε sequence also serves as the origin of the reverse transcription.

The synthesis of the minus strand DNA begins by binding of the Pol protein to the bulge region of the stem-loop structure of the 5' ε sequence.[182,187] This mechanism is supported by evidence that mutations of the bulge region clearly affect the reverse

transcription process.[178,181] The exposed sequence of the bulge region serves as a template for generating a Pol-linked three or four oligonucleotide sequence which serves as a primer for the minus strand DNA synthesis.[185,188,189] Recent studies using a baculovirus expression system for HBV Pol synthesis have shown that the first nucleotide of the minus strand DNA is covalently linked to the 63 tyrosine residue within the TP domain.[190]

The Pol-oligonucleotide primer complex is then translocated efficiently and specifically to the complementary motif within the 3' DR1 region.[187,191,192] This 3' DR1 translocation process is highly specific even though the homologous motifs, complementary to the oligonucleotide primer, are also found in the 5' DR1, DR2 regions as well as other parts of the pregenomic RNA. The specific transfer of the Pol-oligonucleotide primer complex to the 3' DR1 explains the results of earlier studies which showed that the 5' terminus of the minus strand DNA is mapped to the 3' DR1 sequence.[38,193] The mechanism involved in this specific transfer is still enigmatic. It might be influenced by other RNA-protein or protein-protein interactions. It has been speculated that the two ends of the pregenomic RNA may be held close to each other by an unknown circularization mechanism, thus bringing the 3' DR1 region adjacent to the 5' ε sequence.[185]

Once the translocation is complete, synthesis of the minus strand DNA proceeds by reverse transcription with concomitant degradation of the pregenomic RNA template by the RNase H domain of the viral Pol protein.[143,194,195] The minus strand is generated with a short terminal redundancy.[7]

Plus strand DNA synthesis

An important clue for understanding the initiation of the plus strand synthesis has come from the finding that the 5' terminus of the plus strand DNA is mapped to the 3' end of the DR2 region.[38] Synthesis of the plus strand begins after completion of the minus strand DNA and is primed by a capped 15-18 oligoribonucleotide derived from the 5' end degraded product of the pregenomic RNA.[51] The primer sequence includes the 5' DR1 and

six nucleotides upstream. Based on the structural model of reverse transcriptase, if the RNase H domain is located at a distance of ~40 Å from the RT domain, completion of the minus strand will protect the 15-18 nucleotides of the 5' end of the RNA template from the RNase H activity.[196-198]

Translocation of the oligoribonucleotide primer from the 3' DR1 of the minus strand DNA template to the homologous 5' DR2 region initiates synthesis of the plus strand DNA. The mechanism of this primer transfer still remains to be studied but it is possibly facilitated by protein factors. Mutations within the region called the upper binding site (UBS) on the 5' terminal redundancy of the pregenomic RNA have been shown to inhibit plus strand DNA synthesis.[199] This may be associated with the inability of a 65-kD host factor to bind to the mutated site.[199]

Elongation of the plus strand DNA proceeds to the 5' end of the minus strand DNA template until the template is exhausted. An intramolecular template switch then takes place so that the reaction can be continued. It is hypothesized that this template switching is promoted by the presence of the terminal redundancy in the minus strand DNA. Through annealing of the 3' end of the nascent plus strand DNA to the complementary sequence at the 3' end of the minus strand DNA, circularization of the template may be promoted to allow continuation of the synthesis. Physical constraint in the compact nucleocapsid may one of other factors that contribute to the circularization mechanism. The synthesis of the plus strand DNA, however, does not proceed to completion, leaving the characteristic single-stranded gap in the HBV genome.

Viral DNA synthesis has been shown to be absolutely required for viral envelopment and export.[200,201] It has been speculated that genomic DNA synthesis may be coupled with the process of viral envelopment and budding.[172] Mutations in the C terminus of the DHBV capsid protein, although it remains capsid-assembly competent, cause defects in both plus strand DNA synthesis and viral envelopment.[202] Presumably, the plus strand DNA synthesis takes place concomitantly with structural change of the capsid protein

required for nucleocapsid envelopment which completes when a certain stage in the DNA synthesis is reached.[202] As a consequence, mature virions are released without completion of the genomic DNA replication.

Reimport of progeny nucleocapsid DNA: amplification of cccDNA

During the infection, progeny nucleocapsids are reimported into the nucleus, thus leading to amplification and maintenance of the pool of cccDNA. The *de novo* amplification of the nuclear cccDNA was first detected by the experimental infection studies of primary duck hepatocytes with DHBV[216] and also has been identified in HBV infected cells.[203] This cccDNA amplification has been shown to be maintained efficiently in the presence of suramin, a compound blocking DHBV superinfection,[217] a finding which eliminated the possibility that the amplification results from reinfection by released progeny viruses.

The mechanism regulating this intracellular pathway of cccDNA nuclear delivery seems to be linked to the production of the L protein. DHBV mutants lacking the L protein showed greatly enhanced nuclear accumulation of cccDNA.[118,218] The actual mechanism by which the L protein controls the intracellular trafficking of HBV nucleocapsids is still unknown. However, it seems that early in infection, at low levels of the L protein production, the nuclear delivery is preferred. High levels of the L protein accumulated late in infection may stimulate the envelopment of viral nucleocapsids, thus favoring secretion of the particles. Mutations in the DHBV DNA which block virion assembly result in increased cccDNA levels.[219]

Virion assembly and release

Early in infection, the genomic DNA in mature nucleocapsids is reimported into the nucleus, to amplify the cccDNA pool. However, late in infection, an increase in the L protein concentration inhibits the nuclear reimport and, therefore, facilitates envelopment of nucleocapsids.[203,204]

All the viral surface proteins, particularly the L protein, are required for virion formation and release.[115,205] HBV surface proteins, L, M and S subunits, are cotranslationally inserted into the ER where the proteins undergo posttranslational modification, such as glycosylation and myristylation.[206-208] The S and M subunits are secretory proteins mediated by the signal sequence encoded on the S domain.[7] The L protein, however, is not secreted although it contains all the information on the intact S sequence required for secretion.[209,210] This suggests that the preS1 specific sequence may contain signals promoting retention of the L chain. Experimental expression of the L protein also inhibits secretion of the S and M proteins in a dose-dependent manner.[209,211]

In hepadnaviruses, the S protein independently carries out the entire assembly sequence; therefore, this leads to release of unique hepadnaviral subviral particles containing only envelope proteins (mainly the S and M subunits).[7]

HBV virion morphogenesis and secretion seems largely dependent on the function of the L protein.[115,205] This enrichment in L protein on virions supports this hypothesis.[44] Although the mechanism is quite complex and requires further study, it is believed that these processes may be mediated by the cytoplasmic domain of the L protein.[212,213] This domain may act as a matrix protein to facilitate interaction between the envelope proteins and preformed nucleocapsids in the cytoplasm via the capsid protein, thus triggering the envelopment and release of mature virions. This model of the viral nuclocapsid and L protein interaction is supported by mutational analyses of the DHBV preS domain which showed that only those mutants carrying the cytoplasmically disposed preS sequence are competent for assembly.[214]

Assembly of mature HBV virions and subviral particles occurs in the "intermediate compartment" between the ER and Golgi apparatus.[215] Like most eukaryotic secretory proteins, the assembled particles are exported via the constitutive secretory pathway involving the Golgi complex.[69]

II. Gene therapy

2.1 General Principle

Gene therapy, by general definition, involves the introduction of genes into affected cells in order to use their expression for therapeutic purposes. The concept developed from advances in DNA technology, particularly the increased understanding of gene regulation and functions and roles of specific gene products in pathogenesis of human diseases, and the development of effective techniques to transfer DNA into mammalian cells.[220,221]

Human gene therapy protocols employ two general approaches; *ex vivo* and *in vivo* gene transfer. An *ex vivo* gene transfer technique requires removal of the target cells from the host (and probably expansion of the cells in culture), introduction of the desired gene into the cells and subsequently reintroduction of the modified cells into the host. This approach, therefore, is applicable to only a limited range of tissues, for instance liver and bone marrow, which are capable of regenerating.

The fundamental principle of *in vivo* gene therapy involves the introduction of the desired gene directly into the host. This technique avoids the problems associated with the multiple processes of the *ex vivo* approach. However, a suitable and efficient gene delivery vehicle is required to mediate transfer of the desired gene specifically to the target tissues or organs.

2.2 Scope and limitations

2.2.1 Considerations for suitable target diseases

Genetic disorders are the obvious first targets for gene therapy, such as cystic fibrosis,[222] severe combined immune deficiency (SCID) diseases[223] and familial hypercholesterolaemia (FH).[224,225] Several have been investigated extensively in preclinical and clinical trials.

Not all genetic disorders are ideal targets for such therapies. Gene therapy is inappropriate for a disorder involving complicated transcriptional dysregulation, such as β-thalassaemia.[221,226]

For a disease to be suitable for gene therapy, the therapeutic gene must be available and well studied and its delivery to and expression in the relevant tissues must be feasible.[227] Moreover, the effects of the disease must be potentially reversible by gene transfer. So far, gene therapy has been applied to life-threatening diseases, such as SCID[228] and FH,[225] in which the potential risk of serious side effects is ethically acceptable. Cancers and acquired diseases, such as acquired immune deficiency syndrome (AIDS), are other potential diseases to which gene therapy can be applied.[229-230]

The specific aim of gene therapy varies in different diseases. For example, treatment of cystic fibrosis requires the introduction of a new correct copy of the cystic fibrosis transmembrane conductance regulator (CFTR) gene.[233] With cancer, it would be more realistic to deliver a gene coding a toxin to mediate killing of tumor cells, rather than to correct every single genetic defect resulting in malignancies. Any particular application of gene therapy would require consideration of the nature of the functioning gene products, *i.e.* RNA or proteins, type of target tissues and duration of gene expression.[234]

2.2.2 Safety considerations

To date, efforts to develop human gene therapy protocols have focused solely on somatic cells, since the technology is relatively straightforward as compared with germ-cell gene therapy. Ethical concerns regarding the unforeseen problems likely to arise from introducing exogenous material into the gene pool have halted work with germ or reproductive cells as target tissues for gene therapy.[235]

The major concern with respect to potential risks of somatic-cell gene therapy is the possibility of insertional mutagenesis resulting from the introduced gene, particularly when using integrating gene delivery vectors.[236]

2.3 Gene delivery methods

The critical and essential step in gene therapy both *ex vivo* and *in vivo* is the introduction of the desired gene into target cells. As a consequence, the development of methods for the efficient delivery of genes into target tissues with sufficient expression of the required gene products at therapeutic levels has been a major focus for research studies.[237-241] Gene delivery methods which have been developed so far can be divided into two general approaches; physical and biological.

2.3.1 Physical approaches

Physical methods employ mechanical energy to transfer genes across the physical barriers of the cell membrane directly into the cells regardless of cell surface receptors or other features. Physical gene transfer seems to be suitable for delivery of messenger RNA into the cell because the extreme instability of RNA in extracellular environments has made it difficult for transfer by other methods which need manipulation of RNA prior to delivery.[242,243]

At least three different methods of physical gene delivery have been developed, depending on energy sources used for gene transfer. The conventional method is a "direct injection" using needle and syringe to introduce genes directly into the target tissues.[242,244] A "gas-driven gun" has also been employed to propel a solution of DNA into cells.[245] DNA damage has been observed when these methods were used.[246]

The so called "microparticle bombardment" or "gene gun" has been developed to directly deliver DNA into target cells.[243,247-249] This alternative method employs a gene gun

to generate "a defined electrostatic discharge and a subsequent shockwave"[246] to deliver nucleic acid-coated gold particles in high velocities across the membrane barrier into cells. The efficiency of gene transfer depends primarily on the number of particles introduced and the ratio of the amount of nucleic acid to gold.[247] Depth of penetration is dependent on the particle size and the discharge voltage.[246] The method seems to be more efficient than the other direct gene transfer procedures, since lower quantities of nucleic acid are required to produced an equivalent transfection.[250] This gene delivery approach can be applied with various somatic cell types both *in vivo* and *in vitro*.[243,249]

The actual mechanism of the nucleic acid uptake by these methods is unclear but it does not seem to be associated with direct cell injury.[251] Expression of genetic information transferred by these physical approaches is usually transient if the nucleic acids cannot exist as episomes or become integrated.

2.3.2 Biological approaches

Viral vectors

Two distinctive characteristics that make viral vectors the most studied gene delivery systems are their efficiency in delivering nucleic acid to particular host cells and their ability to evade the host immune system. Recombinant viral vectors are generally composed of a modified viral genome carrying the desired gene surrounded by the virion structure which is necessary for cellular recognition and entry. These vector particles are generated from two basic components: a vector DNA plasmid and a helper or packaging construct.[252] Ideally, the vector plasmid will contain only the essential viral sequence required in *cis* and the transcription unit of the gene of interest. All nonessential viral sequences which can be provided in *trans* should be removed. All these functions, which are needed for replication and assembly of the vector genome into recombinant vector particles, will be supplied in *trans* by a specific packaging or helper cell line. Therefore, ideal viral vectors should

express only the exogenous gene in the target cells without viral replication or gene expression. Expression of viral proteins by the viral gene remaining on the vector may cause cytotoxicity[253] or lead to stimulation of an immune response against the target cells which may result in decreased effectiveness.[254,237] Viral vectors introduce the desired gene into target cells by the same process as their natural infection pathway by specific recognition on the target cell receptor.

To date, several viruses have been modified to be efficient gene transfer tools.[252] These systems have different features, for instance, the capacity to accommodate a foreign gene, tissue or organ specificity, and cellular retention. Therefore, each of these vectors will probably be suited for individual applications of gene therapy that will be determined by the target tissue, the size of therapeutic genes to be delivered and the required duration of gene expression.

Retroviral vectors

Most retroviral vectors presently used in gene therapy are derived exclusively from murine leukemia virus (MLV).[255,256] Generally, an MLV-based retroviral vector plasmid contains the essential *cis*-acting sequences, including the 5' and 3' long terminal repeats (LTR) and a packaging sequence, and a transcriptional unit of an exogenous gene.[252,255,256] The LTR sequences contain essential viral functions involved in replication and integration of the vector DNA. The packaging signal, which encompasses the sequences near the 5' LTR and the N-terminal *gag* coding sequence, is required for encapsidation of the vector DNA into virus particles.[255] However, recent studies have suggested that the *gag* coding sequence and the immediate upstream sequence could be removed from the vector constructs without affecting the packaging efficiency[257] Expression of an exogenous gene is normally controlled by the viral LTR. Addition of a heterologous internal promoter has proved to be problematic since interference between heterologous promoters and the LTR has been described.[258-260] Interference between promoters in retroviral vectors, however,

may be avoided by modifying or replacing the retroviral promoter. For example, deletion of the enhancer element in the U3 region of the 3' LTR has been shown to alleviate the inhibitory effects of the retroviral promoter on internal promoters.[261] A therapeutic gene replaces all the retroviral coding sequences; therefore, the vector constructs are replication-defective. Up to about 10 kb of a vector genome can be efficiently packaged.[252] The missing viral functions are supplied in *trans* by a packaging cell line.[255] The engineered packaging cell line contains retrovirus-derived sequences for synthesis of authentic viral structural proteins but lacks a packaging signal; therefore no helper viral sequences become packaged.[262]

The major advantage of MLV-derived retroviral vectors are the high efficiency of gene transfer and the ability to integrate into the host genome making them capable of long-term expression. The apparent inability to infect nondividing cells is an important drawback in the use of these vectors.[263] Moreover, MLV-derived vectors packaged in murine packaging cell lines appear to be susceptible to human complement-mediated inactivation *in vivo*.[264,265] The ability of these retroviral vectors to infect a wide host range also results in the problem of unintended transduction of nontargeted cell types. Modification of the viral envelope proteins by replacement with specific cellular targeting ligands or generation of pseudotype viral envelopes has been shown to improve the tissue specificity of the vectors.[240,256]

Two potential problems regarding the safety of these vectors include the risk of insertional mutagenesis due to random integration of the vector DNA and possible contamination with helper virus particles produced by homologous recombination of the vector sequence and the retroviral sequence carried by the packaging cell line.[236,256] Different generations of retroviral vector systems have been developed to minimize the possibility of homologous recombination.[256]

Attempts have been made to develop other retroviruses, including human immunodeficiency virus (HIV), as gene delivery systems.[266,267] However, because the genomic structure and gene regulation mechanism of HIV are complicated, these vectors

have been more difficult to construct than have MLV-derived vectors.[266,268] HIV-based retroviral vectors, however, would have several advantages, including the ability to infect nondividing cells and to deliver genes preferentially to CD4 bearing T cells.[256]

Adenoviral vectors

Adenoviruses are nonenveloped viruses; therefore, they are more stable and less sensitive to complement-mediated inactivation than enveloped viruses.[269] Virtually, all adenoviral vectors are E1-deletion vectors and retain the immediate 5' end of the viral genome, including the terminal repeats and packaging sequence.[252,269] Since E1-encoded functions are essential for viral replication, packaging of the E1-replacement vectors occurs only in a complementing cell line which constitutively expresses E1 proteins, *i.e.* 293 cells.[270] Some adenoviral vectors have been derived from E1 and E3 deletion recombination since the E3 gene has no effect on adenoviral infectivity.[222,271,272] Such E1/E3 deletion vectors have a maximum cloning capacity of approximately 8 kb.[271,272] To clone larger fragments, other viral coding sequences, such as E2 and E4, can be deleted from the vector DNA and the missing functions are provided in *trans* by a complementing cell line or helper virus.[273-275] Attempts to create adenovirus vectors by removal of all the viral genes, except the inverted terminal repeats and the packaging signal, have been reported.[276,277] These vectors are able to accommodate over 30 kb of foreign DNA. However, the yields of the purified vector particles were low in comparison with yields obtained from the E1/E3 deletion vectors.

The major advantages of adenoviral vectors are their ability to propagate the vector particles in very high titer and capability to infect nondividing cells. Since adenoviruses are capable of infecting a variety of cell types, tissue specificity is one of the important drawbacks related to use of adenoviral vectors. Similar to the retroviral vector systems, tissue-specific promoters and cell surface-specific ligands have been incorporated into the adenoviral vector contexts for restricted gene expression.[278-280]

Another major drawback of adenoviral vectors is the elicited host immune response against the viral proteins which may be responsible for transgene instability or decrease in effectiveness.[237,254,281,282] Production of replication-competent helper viruses due to homologous recombination during the vector preparation can also occur.[283] Recent development of a helper-dependent adenoviral vector system has utilized a *cre-lox* system to minimize the potential of helper virus production.[284] By this strategy, helper viruses are constructed with packaging signals flanked by *lox*P sites so that in the 293 cells which stably express the Cre recombinase (293Cre cells) the packaging sequence is excised, thus rendering the helper virus genome unpackageable. Only the vector DNA can be packaged. Since all the functions necessary for virion formation are provided by the helper virus, the majority of the adenovirus vector genome can be replaced with a large foreign gene.

Adeno-associated viral vectors

Adeno-associated virus (AAV) is a nonenveloped virus in which a productive infection requires coinfection with certain viruses, usually adenoviruses.[285] In the absence of helper virus coinfection, AAV integrates into the host genome. The integration site of wild type AAV appears to be site-specific at chromosome 19q13.3-qter.[286]

AAV-based vectors have been developed using several strategies.[287-289] All of the vectors, however, contain the viral inverted terminal repeats (ITRs) flanking a transcription unit of an exogenous gene.[289] The ITRs serve as the origin of the viral genomic DNA replication and are necessary for virion encapsidation and host chromosomal integration.[288,290] They also possess intrinsic promoter activity.[290] The size of the exogenous DNA to be expressed by AAV vectors is limited since vectors greater than 115% of the wild type genome size (4.7 kb) are generally not packaged efficiently.[287] Expression of an exogenous gene by these vectors is controlled either by endogenous AAV transcriptional regulatory sequences or using heterogeneous promoters.[289] In one

approach, expression of the large transgene, CFTR, by this type of vector has been regulated by the ITRs themselves.[290]

AAV vectors are doubly defective in replication; *i.e.* requisite AAV-encoded regulatory and structural proteins have been replaced by the cassette of exogenous gene and they are lacking the functions from a helper virus necessary for productive replication. AAV vector particles are generated by cotransfection of the vector DNA plasmid with an AAV-packaging plasmid, lacking ITRs but encoding AAV-protein functions, into adenovirus-infected cells or helper cells.[287,288] The resultant products of this replication and packaging system are AAV vector particles and helper adenovirus particles. Although adenovirus virions are subsequently removed by physical separation approaches, the risk of contamination with the helper particles is still a major concern in the development of AAV vectors. Xiao and colleagues[291,292] have recently constructed a novel AAV-helper system in which helper virus functions are provided by cotransfection of a mini-adenovirus genome capable of propagating AAV vector particles and an AAV-packaging plasmid. Infectious helper adenoviruses would not be produced because this mini-adenovirus genome does not encode some of the early and late adenoviral genes which are essential for propagation of adenovirus particles.

The major potential advantages of AAV vectors are their apparent capability for long-term expression in nondividing cells due to integration and their lack of human-associated pathogenicity.[293]

Other viral vectors

Additional mammalian viruses which are under development or have a potential for use as vector systems for gene therapy include herpes simplex virus (HSV),[253,294,295] autonomous parvovirus,[289,296] and poliovirus.[297]

Of these viral vectors, HSV is the most extensively studied. Due to its natural infection of neurons of sensory ganglia and ability to establish a life-long quiescent "latency" state

without stimulation of host immunity, HSV-derived vectors could serve as gene delivery systems for therapy in the nervous system. However, because of the complex regulation and genomic organization of HSV, development of these vector systems has been problematic.[253,295] The major problem is the cytotoxicity of HSV vectors, although they are replication-incompetent, which is probably due to the expression of the viral protein products of the viral genes remaining on the vector.[253] Further development of safe and efficient HSV vectors will involve deletions of replicative and pathogenic functions from the vector genome and the use of a latency active promoter to stably control expression of the transgene from the defective virus DNA.[253,295]

Receptor-mediated gene delivery systems

This approach is based on the receptor-mediated endocytosis pathway to specifically target DNA into specific tissues. The process involves generation of complexes of negatively charged DNA molecules and polycationic polymer, *i.e.* poly-L-lysine, covalently linked to polypeptide ligands, that can be recognized by specific receptors on the cell surface.[298,299] An initial study using this approach was performed by Wu and Wu[300] who have directed gene transfer to the liver by targeting the asialoglycoprotein receptor uniquely expressed on the hepatic cell surface.

One major drawback related to this gene delivery system is that the endocytic vesicles formed during this process are normally transported to the lysosome where the contents of the endosome are degraded.[301] Therefore, effective gene transfer by this procedure depends on the escape of DNA from this degradative pathway. Improvement of gene expression has been achieved by addition of endosomolytic agents, such as defective adenovirus particles,[302,303] the hemagglutinin HA-2 protein of influenza virus[304] or lytic peptides,[305] to induce efficient disruption of DNA containing endosomes.

For effective gene delivery, it is essential that the DNA complexed particles are condensed into a nanoparticulate structure. The suitable effective size of these particles is

dependent on the target organ;[238,306,307] however, the final size of the compacted DNA is dictated by the size of the original plasmid construct as well as the properties of the polycationic material and associated ligands.[238] A DNA complexed particle of 10-12 nm in diameter seems to be taken up efficiently and gives persistent gene expression in the liver.[307]

Liposome-mediated gene delivery systems

Liposome-mediated gene transfer has made use of lipids to deliver a vector DNA into target cells.[240,308] A conventional liposomal system has been designed to incorporate vector DNA within a lipid membrane.[308] In the early efforts, phosphatidylserine, a lipid bearing a net negative charge, was used as a major component of the membrane.[309,310] This conventional liposomal system cannot be flexibly applied for targeted-gene delivery purposes since it is selectively taken up by cells of the reticuloendothelial systems, particularly macrophages residing in liver, spleen and bone marrow, because of their limited extent of extravasation.[240,311] The efficiency of gene transfer is also dependent on the ability of the DNA to escape from the lysosomal degradative pathway. pH-sensitive lipids have been incorporated into the encapsulating liposome to improve endosomal disruption and entry of the liposomal contents into the cytoplasm.[312]

Polycationic lipids are further modified lipid formulations which allow direct fusion of liposomes and cell membranes and hence efficiently avoid the lysosomal pathway.[313,314] Electron microscope evidence has suggested that polycationic lipids may coat the DNA, thus producing a cationic shell which is optimal for interaction with negative charges on the cell surface.[315] A series of studies in animals and humans of both local and systemic administrations suggests that these liposomal complexes are nonimmunogenic, do not have significant toxicity and can be administered repeatedly with expectations of equivalent gene transfer.[316-320] Expression of exogenous genes by polycationic-liposomal systems has been

demonstrated in many tissues, including lung, liver, spleen, bone marrow, heart and lymph nodes.[321,322]

Current liposome formulations do not restrictively target gene to specific tissues. Tissue specificity of liposome-mediated gene transfer has been achieved by incorporating DNA with tissue-specific promoters to target gene expression (see below) or coupling with cellular specific ligands to mediate specific gene delivery.[308,322,323] For example, incorporating a triantennary galactolipid into the polycationic lipid-DNA complex has targeted gene transfer to hepatocytes through interaction with the asialoglycoprotein receptor.[323]

Tissue-specific regulatory elements as gene targeting systems

Tissue-specific promoter/enhancer elements are incorporated into plasmid or viral vectors to develop tissue-specific gene delivery systems by which expression of the therapeutic gene is restricted to desirable cell types. The most pragmatic approach is to use the cellular promoter/enhancer elements native to the target tissues. Promoter/enhancer sequences of some viruses have been developed for targeting gene expression according to their host-cell tropism, such as the development of HBV promoters/enhancers for specific gene expression in the liver.[324] A short summary of tissue-specific regulatory elements which have been developed for tissue-directed gene transfer is presented in Table 1.2.

To be able to obtain both tissue-specificity and controllable time and duration of gene expression, inducible promoters have been also developed.[325,326] One of the most studied examples of such systems is the tetracycline-controlled expression system in which expression of a therapeutic gene is activated in the presence of tetracycline to one thousand fold over the level seen in the absence of the drug.[327,328] However, the drawback with systems of this type is that three agents, *i.e.* the therapeutic gene, the transcriptional activation gene and the drug used to induce transcription, must all be delivered to target cells.

Table 1.2 Tissue-specific regulatory elements for tissue-directed gene expression

Promoters/enhancers	Target tissues	References
Insulin promoter	β-islet cells of the pancreas	329
Elastase promoter	Acinar cells of the pancreas	330
Whey acidic protein promoter	Breast	331
Tyrosinase promoter	Melanocytes	332
Ren-2 promoter	Kidney	333
Collagen promoter	Connective tissues	334
α-actin promoter	Muscle	335
α-fetoprotein promoter	Liver	336
Albumin promoter	Liver	336
Uroplakin II promoter	Bladder	337
Immunoglobulin heavy chain promoter	B lymphocytes	338
Protamine promoter	Testes	339
HBV C promoter/enhancer I	Liver	324
Human α1-antitrypsin promoter	Liver	340, 341

Extrachromosomal replicating vectors

Persistence of gene delivery vectors carrying a therapeutic gene in target cells is desirable in most applications of gene therapy, which require long-term gene expression or when target cells are undergoing proliferation. Besides integration of vectors into the host chromosome, vector longevity can be achieved by the ability to replicate and be maintained extrachromosomally. A series of such vectors, including an artificial human chromosome, an Epstein-Barr virus (EBV)-based vector and human chromosomal *ori* vector, are under development by utilizing either chromosomal or viral elements to mediate replication and nuclear retention.[241,342]

An artificial chromosome is a theoretically desirable vector which would consist of native mammalian chromosomal components required for replication, retention and stability, including an origin of replication, a centromere and telomeres.[241] However, construction of such an artificial chromosomal vector has not yet been possible. The inclusion of the minimal size of a functional centromere would render the vector size unacceptably large, at least 1 Mb, which would pose problems in construction, manufacture and delivery of the vector to target cells.[343]

An EBV-based vector employs *cis*-acting sequences and *trans*-acting factors of human EBV for replication and retention in the nucleus. This type of vector is composed of the EBV *oriP*, consisting of the EBV family of repeats and the dyad symmetry element, and the viral *EBNA-1* gene.[342,344] The *trans*-acting gene product of the *EBNA-1* gene, required for activation of the origin of replication and for the stable nuclear retention of the vector, binds to both regions on *oriP*.[345,346] An EBV-based vector is about 6 kb in size, in which exogenous genes can be inserted up to several hundred kilobases.[347,348] The vectors can be delivered by liposome-mediated methods[349] or packaged into an EBV viral capsid, in which case vector delivery is restricted to natural host cells of EBV, predominantly human B lymphocytes.[350] Expression of the CFTR gene by an EBV-based vector has been

demonstrated in transformed, dividing human airway epithelial cells for about two months.[351] An EBV-based vector, however, has limited host range for replication due to the presence of the EBV *oriP* sequence.[345,352]

Another type of extrachromosomal replicating vector, a human chromosomal *ori* vector of 22 kb, has been modified from the EBV-based vector.[241] This system contains the human genomic *ori* sequence to mediate replication and the EBV family of repeats and the viral *EBNA-1* gene to maintain the vector in the nucleus.[241] Using the human origin of replication allows this vector to replicate in all mammalian cell types.[241,352]

The requirement for the *EBNA-1* protein for the replication and stability of these vectors, however, leads to the possibility that the protein might protect the target cell from the immune response elicited by the therapeutic gene since the *EBNA-1* protein seem to be able to evade the host immune response.[350]

2.4 Gene therapy and liver diseases

The liver plays a central role in homeostasis, metabolism and protein synthesis; therefore, it is a major site for manifestation of several inherited metabolic diseases, such as FH and hemophilia B.[353-355] Although many of these diseases are well characterized, effective treatments are not available. The advent of gene therapy provides promise for future treatments for these liver diseases, as well as other acquired liver diseases such as malignancies.[355-357] Both *ex vivo* and *in vivo* gene transfer approaches have been investigated for liver-directed gene therapy.[358,358] Hepatocyte-directed *ex vivo* gene therapy has been performed in patients with FH,[225] a disease that results from a defect in the low-density lipoprotein (LDL) receptor gene in hepatocytes.[359] In these studies, hepatocyte cultures established from the patients' liver cells were transduced with recombinant retroviral vectors that contained a functional LDL receptor gene. The cultures with

evidence of efficient gene transfer were reintroduced into the patients via the portal veins. During the eighteen month period of treatment, the patients showed stable improvement.[225]

Although much progress has been made in liver-directed gene therapy *ex vivo*, the cumbersome technical and clinical procedures related to the excision, cultivation and reimplantation of hepatocytes have led to studies on the development of *in vivo* gene transfer approaches. These latter strategies are also more suitable for long-term treatment.[360] The most crucial requirement for effective *in vivo* gene therapy for liver diseases is the availability of efficient vectors to target gene delivery and expression only in hepatocytes.

Current viral gene transfer systems are not ideal for liver-directed gene therapy *in vivo*. Retroviral and adenoviral vectors, though apparently the most efficient gene delivery systems, possess broad host ranges which lead to undesirable gene transfer to nonhepatic tissues. Attenuation and/or inactivation of the retroviral LTR have been described for *in vivo* gene transfer in hepatocytes.[361] Moreover, adenoviral vectors have been reported to poorly infect primary hepatoma cells *in vivo*.[362,363]

Several studies have shown that genes of interest can be delivered specifically to liver cells using receptor-mediated gene delivery systems to target receptors uniquely expressed on the liver cell, such as the asialoglycoprotein receptor.[300,364,365] Use of tissue-specific promoters in combination with viral vectors have been shown to improve liver-targeted gene transfer *in vivo*.[278,366] Several promoter/enhancer elements of cellular genes, including human α1-antitrypsin promoter,[340] human apolipoprotein E enhancer,[340] mouse albumin promoter[278] and human α-fetoprotein promoter,[366] which function mainly in the liver have been studied for liver-directed gene therapy *in vivo*.

III. Rationales and objectives

The marked hepatotropism of HBV makes it an attractive candidate for gene delivery vector for liver-directed gene therapy *in vivo*. Its liver specificity is governed by recognition of specific receptors present on the cell surface and requirement for liver-specific factors for regulatory functions of HBV promoter and enhancer elements.[39-41] HBV-derived promoters have been evaluated for use in liver gene therapy and expression from these promoters has been shown to be sustained for at least two months in hepatocyte-derived cell lines.[367] These HBV-derived promoters also retain their activity and liver specificity in driving expression of the LDL receptor gene in the context of adenoviral vectors.[324] To our knowledge, however, HBV has not been developed as a viral vector for gene transfer.

The overall goal of this research was to develop HBV as gene delivery systems for gene therapy of liver diseases. The study began with investigations of the possibility to develop HBV as one or more gene delivery vectors. Since the HBV genomic organization is very compact and contains overlapping ORFs, disruption or replacement of HBV genes with a foreign gene could interfere with its replication and gene expression. In this first study, we therefore inserted the HIV-1 *tat* gene, used as a reporter, into the dispensable tether region in-frame with the P gene. With this approach, none of the HBV ORFs would be disrupted and this type of vector was expected to be replication-competent. Expression of the foreign gene in the HBV context, effects of the insertion on HBV gene replication and expression as well as ability of this HBV vector to form the recombinant vector particles were determined.

We then tested the possibility that a gene larger than HIV-1 *tat* could be expressed by the replication-competent HBV vector using the Zeocin™ resistant gene (Zeo^R) as a reporter. The development of an HBV replication-defective vector was also investigated.

We next assessed the expression of a therapeutic gene by HBV vectors. Expression of the chicken anemia virus VP3 (CAV-VP3) or apoptin gene, a candidate for cancer gene

therapy, was determined in both replication-competent and replication-incompetent HBV vectors.

Since the most important property of viral vectors for gene therapy is the ability to transduce target cells, the infectivity of the HBV recombinant vector particles also needed to be determined. Because a reliable protocol for determination of HBV infectivity *in vitro* is not available, we had to test a published protocol for an HBV infection assay[368] prior to testing for infectivity of the vector particles.

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CHAPTER 2*

Development of a replicative hepatitis B virus vector: demonstrating the expression of the functional HIV-1 *tat*

I. Introduction

As we are faced with a number of diseases involving liver, particular inherited single gene defects and viral diseases, a novel therapeutic approach using targeted gene transfer to this organ is of particular interest, especially, strategies of using human viruses as vectors. *In vitro* protocols for transferring the low density lipoprotein (LDL) receptor gene into hepatocytes using a retroviral vector have been established.[1,2] Adenoviral vectors have also been used to deliver therapeutic genes, such as the genes for factors VIII[3] and IX[4,5] and LDL receptor[6,7] into liver cells. However, these viral vectors infect a wide range of tissues, not specifically targeting to the liver. Expression of the transferred gene by adenoviral vectors, for example, is detected in different tissues after systemic administration[6,8,9] or via portal or splenic vein.[7,10,11] Therefore, the use of hepadnaviruses, which are hepatotropic and possess strong liver specific promoter and enhancer elements,[12] as vector systems may provide a more efficient mean for gene delivery to the liver.

Hepadnaviruses are amongst the smallest DNA viruses known, carrying only 3,200 base pairs in their genome. The genomic organization of these viruses is extremely compact and efficiently organized with overlapping open reading frames (ORFs).[13,14]

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Hepatitis B virus (HBV), the prototype of hepadnaviruses and causative agent for human hepatitis, carries four major overlapping ORFs: preS1/preS2/S (collectively known as the envelope or surface gene), preC/C, X and P. The envelope gene, encompassing the preS1, preS2 and S regions as delineated by three in-frame initiation codons, codes for three envelope proteins: large (L), middle (M) and major (S). The preC/C ORF has two in-frame initiation codons for the preC and C genes which code for secreted HBV e antigen (HBeAg) and capsid or core protein (HBcAg), respectively. The X gene codes for a transactivating protein which has activity on HBV enhancers and other cellular genes.[15] The P or polymerase gene has the longest ORF. It encompasses about 80% of the entire viral genome and overlaps with the C-terminus of the preC/C gene, the entire envelope gene and the N-terminus of the X gene. The C-terminus of the X gene also overlaps with the N-terminus of the preC/C gene. The protein product (Pol) encoded by the P gene can be divided into three major functional domains: the terminal protein domain at the N-terminus, the reverse transcriptase/DNA polymerase in the central domain and the RNase H domain at the C-terminus.[16,17] The terminal protein and reverse transcriptase/DNA polymerase domains are separated by a spacer or tether region. Four promoter elements, the preS1, preS2/S, X and C or C/P promoters,[18] which regulate transcription of genomic and subgenomic messengers for expression of the corresponding genes, have been identified on the HBV genome. Almost all the nucleotides are included in coding sequences and are therefore indispensable. Only the spacer or tether region may be non-essential for the P gene function or HBV replication.[17,19]

To my knowledge, HBV or other hepadnaviruses have not yet been engineered and used as gene transfer tools. The unusually efficient genome of HBV is a factor that limits its genetic manipulation. Mutations, insertions or deletions in many regions of the HBV genome have deleterious effects on viral gene expression and replication.[17,20-24] The tether

region of the P gene, however, seems to be dispensable for HBV replication and can be manipulated. Mutational and computer sequence analyses show that this region starts upstream of the preS1 gene and overlaps with the preS1 and preS2 regions.[17,21] Part of the tether region, however, does not overlap with any other HBV genes. A mutational analysis of the P gene of HBV has demonstrated that up to 90 codons of the intervening tether sequence can be deleted without significant loss of the endogenous polymerase activity.[17] It has also been shown that such a deletion has no effect on the RNA encapsidation process.[25] Mutants of HBV containing deletions in the preS1 region which overlaps the tether region are capable of replication.[23] The duck hepatitis B virus (DHBV) genome carrying the gene for protein A (123 amino acids) inserted in the tether region also retains the capability of expressing an active endogenous polymerase.[19] This region, moreover, tolerates many mutations resulting in amino acid changes.[26,27] The tether region, therefore, seems to be the most suitable site for engineering the HBV genome as a vector.

In this study, I have successfully manipulated the HBV genome to accommodate a foreign gene whose functional activity can be demonstrated in the context of the full length HBV genome in tissue culture cells. A replicative HBV vector carrying the human immunodeficiency virus type 1 (HIV-1) *tat* gene in the tether region was constructed. Transient expression in hepatoma and cervical carcinoma cells showed that the *tat* gene was expressed with functional activity. This HBVtat recombinant exhibited polymerase activity, albeit at a reduced level compared to the wild type HBV. Remarkably, intact viral particles were still produced from human hepatoma cells transfected with the HBVtat recombinant.

II. Materials and Methods

Plasmid construction and mutagenesis

A pTHBV plasmid was constructed by subcloning the full length genome (*Eco*RI-*Eco*RI) of HBV adw2 subtype in the pT7T318U vector (Pharmacia Biotech, Uppsala, Sweden). A 267-base pair (bp) HIV-1 *tat* cDNA fragment with additional *Bst*EII sites at both ends was amplified from plasmid pCEP-*tat*[28] by PCR using the upstream primer 5' TGCGGGTCACCAATGGAGCCAGTAGATCCTAAT 3' and the downstream primer 5' ATATGGTGACCCTTCCGTGGGCCCTGTCGGGTC 3' (the *Bst*EII sites are underlined). The Pfu polymerase (Stratagene, La Jolla, CA, USA) was used to minimize the error rate of the polymerase. The PCR amplified *tat* fragment was subcloned into the unique *Bst*EII site in the P ORF of the HBV genome in pTHBV. The HBV genome carrying the *tat* gene is designated HBVtat. DNA sequencing confirmed the actual sequence. Replication-competent plasmids of wild type HBV (pTHBV-d) and HBVtat (pTHBVT-d) were constructed by ligation head to tail of two copies of the full length HBV (*Eco*RI-*Eco*RI) sequence and HBVtat (*Eco*RI-*Eco*RI) sequence, respectively, in the pT7T318U vector. The expression of these replication competent HBV plasmids in an eukaryotic system is controlled by the HBV endogenous promoters.

Mutations of the X gene of HBVtat were performed by site-directed PCR mutagenesis. Three oligonucleotide primers were designed. The upstream primer 5' TTACTAGTGCC-ATTTGTTCAGTGGTTCG 3' was homologous to the sequence at the unique *Spe*I site (underlined) located 142 bp upstream of the X gene. The downstream primer 5' GTG-CACACGGACCGGCAGATG 3' anneals to the sequence at the unique *Rsr*II site (underlined) located 197 bp downstream of the X gene. The mutated oligonucleotide 5' ATACATCGTTTCCcTGGCTGCTAGGCTGTACTGCtAACTGGATCCTTC 3' was targeted to the sequence at the unique *Nco*I site (underlined) at the initiation codon of the X

gene with change from A to C at the 1376 nucleotide (nt) and from C to T at the 1397 nt. These changes abolished the initiation codon of the X gene and the original *Nco*I site with the addition of a stop codon (mutated nucleotides shown in boldface lower cases). These mutations conserved the P coding sequences. The mutations were performed by multiple PCR as described below. The mutated PCR fragment was then cut with *Spe*I and *Rsr*II and cloned into the unique sites in the HBVtat plasmid. A frameshift mutation of the P ORF of HBVtat was generated by opening at the unique *Bsp*EI site (2331 nt) downstream of the initiation codon of the P gene and subsequently filling in (2332 to 2336 nt) with Klenow Fragment (GIBCO BRL Life Technologies, Gaithersburg, MD, USA). The mutation disrupted the reading frame of the P gene. It, therefore, ablated the expression of the *tat* insert as a Pol-Tat fusion recombinant. These mutated sites were verified by restriction mapping and DNA sequencing.

To construct a HBV core expression plasmid, pCHBVC, a 1500 nt fragment from the *Nla*III site to the unique *Avr*II site which includes the entire sequence of the core gene was PCR-amplified from the HBV genome containing plasmid, pKSVHBV1[29] and cloned into the pTZ19R vector (Pharmacia Biotech). The sequence between the *Hin*dIII and *Xba*I sites containing the core gene was subcloned into the eukaryotic expression vector pcDNA I Amp (Invitrogen, San Diego, CA, USA). An HBV P plasmid (pCHBVP) was constructed by subcloning a 2734-bp fragment containing the entire P ORF from pKSVHBV1 into the pTZ19R vector by multiple cloning steps using restriction enzymes and PCR. The sequence coding for the entire HBV P ORF was cut and subcloned into the *Hin*dIII/*Eco*RV sites of the eukaryotic expression vector pcDNA I Amp. The subcloned sequences of these recombinant plasmids were verified by restriction mapping and DNA sequencing. pSG-X was constructed by inserting the X gene into the *Eco*RI/*Bgl*II sites of the eukaryotic expression vector pSG5 (Stratagene). A pSV45 plasmid carried the entire HBV surface antigen ORFs for the simultaneous expression of L, M and S surface proteins.[30] pCEP-*tat*

carried the *tat* gene under the CMV promoter.[28] pLTR-CAT is a CAT reporter plasmid carrying the CAT gene under the HIV-1 LTR.[28]

PCR subcloning

The protocol was modified for subcloning a short DNA fragment (~250-500 bp). PCR reaction was performed on a Single Block™ System (Ericomp Inc., San Diego, CA, USA). A 100 µl total volume of PCR reaction contained 20 ng template DNA, 0.5 µM each of upstream and downstream primers, 50 µM each of dNTPs and 2.5 units Pfu polymerase (Stratagene) in 1X Pfu polymerase buffer (Stratagene). After thoroughly mixing the other components, the enzyme was added last. The reaction mixture was then overlaid with mineral oil. The PCR reaction was performed at one cycle of 95°C, 1 min, followed by 30 cycles of 95°C, 1 min; 56°C, 1 min; 72°C, 45 sec. The PCR products were purified using 30 000 NMWL MC Ultrafree® filters (Millipore Corporation, Bedford, MA, USA) and ready to be used for subcloning. The subcloned sequence was verified by DNA sequencing.

Site-directed PCR mutagenesis

Site-directed PCR mutagenesis required three oligonucleotide primers: two flanking primers, which were upstream and downstream of the mutation site, and one mutagenic primer at the mutation site. PCR reaction was performed on a Single Block™ System (Ericomp Inc.). The procedure, which involved three-step PCR reaction, was performed as described by Picard *et. al.*[31] with some modifications.

Step 1: This initial step involved amplification of the mutated fragment. PCR reaction was carried out at 95 µl total volume which contained 15 ng template DNA, 100 nM each of mutagenic and downstream primers, 200 µM each of dNTPs and 2.5 units Pfu polymerase (Stratagene) in 1X Pfu polymerase buffer (Stratagene). After thoroughly mixing the other components, the enzyme was added last. The reaction mixture was then

overlaid with mineral oil. The amplification was performed at ten cycles of 95°C, 1 min: 56°C, 1 min; 72°C, 2 min, followed by one cycle of 72°C, 5 min and holding at 4°C.

Step2: The upstream primer was added to the reaction mixture: 2.5 µl of a 20 µM solution per reaction. After thoroughly mixing, the reaction mixture was subjected to the same amplification as described in Step 1.

Step3: The downstream primer was added to the reaction mixture: 2.5 µl of a 20 µM solution per reaction. After thoroughly mixing, the reaction mixture was again subjected to the same amplification as described in Step 1.

After removal of mineral oil, the PCR products were purified using 30 000 NMWL MC Ultrafree® filters (Millipore Corporation). The mutated sequence was verified by DNA sequencing.

DNA Sequencing

Double stranded DNA sequencing was performed using a SEQUENASE Version 2.0 Sequencing Kit [United States Biochemical Corporation (UBS), Cleveland, OH, USA] with some modifications as described.

Alkaline denaturation and annealing reaction of DNA template: DNA was prepared by alkaline-lysis minipreparation method and dissolved in 15-25 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5-7.6) depending on the DNA yield. A 5 µl aliquot of the DNA was used as DNA template. A sequencing primer (1µl of 10 ng/µl concentration) was added, followed by addition of 1 µl of 1M NaOH. The solution was mixed pipetting and incubated at 37°C for 10 min. One microliter of 1M HCl was added and mixed by pipetting, followed by addition of 2 µl of 5X Sequenase Reaction Buffer. The mixture was further incubated at 37°C for 5 min and kept on ice for subsequent reaction.

Preparation of termination mixtures: While the annealing mixture was incubating, 4 tubes of each termination mixture were prepared in terizaki plates, each containing 2.5 µl of ddATP, ddCTG, ddGTP or ddTTP. The plate was then prewarmed at 37°C.

Labeling reaction: Prior to performing the labeling reaction, 1 µl of Sequenase enzyme was diluted with 0.5 µl of Pyrophosphatase and 6.5 µl of Enzyme dilution buffer. The diluted enzyme was kept on ice and used within 1 hr. The Labeling mix was diluted 5 fold with distilled deionized water (ddH_2O).

To an ice-cold annealing mixture, the following solutions were added: 1 µl of 0.1 M DTT, 2 µl of the diluted Labeling mix, 0.5 µl of [^{35}S]dATP (1000 Ci/mmol) (Amersham Life Science, Oakville, ON, Canada) and 2 µl of diluted enzyme. The labeling reaction was incubated at room temperature for 5 min.

Termination reaction: To the prewarmed termination mixture in each well, 3.5 µl of the labeling reaction was added, mixed and incubated at 37°C for 5 min. The reaction was stopped by adding 4 µl of the Stop solution. The completed reactions were stored -20°C until further analysis.

Gel electrophoresis: The sequencing reactions were analyzed by 8% urea-acrylamide gel electrophoresis. The samples were heated at 70-75°C for 5-10 min before loading. After fixing in a 10% methanol and 10% glacial acetic acid solution and drying, the gel was analyzed by autoradiography.

Cell culture and transfection

Human hepatoblastoma (HepG2) and human cervical carcinoma (HeLa) cells were cultured and maintained at 37°C in 5% CO_2 in Auto-Pow MEM Eagle (modified) medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 10 mM sodium bicarbonate, 2 mM L-glutamate, 10% fetal bovine serum (FBS), 50 units/ml penicillin G sodium, 0.01 mg/ml streptomycin and 50 units/ml nystatin. Avian hepatoma cells (LMH) were cultured

and maintained at 37°C in 5% CO_2 in a mixture of 1:1 Auto-Pow MEM Eagle (modified) and F12 (ICN Biomedicals Inc.) media with the same supplementation as the HepG2 medium.

Transfections were performed using Lipofectin (GIBCO BRL Life Technologies) for HepG2 cells and Lipofectamine (GIBCO BRL Life Technologies) for LMH and HeLa cells using the procedure recommended by the manufacturer with some modifications. Cells were subcultured in 60-mm tissue culture dishes 20 hr prior to transfection and were about 60-70% confluent at the time of transfection. Cells were fed with fresh media 1 hr before transfection. The plasmid DNA and Lipofectin or Lipofectamine (1:6 ratio) were each diluted into 300 µl of unsupplemented medium (no FBS and antibacterial agents). These two solutions were combined, incubated for 15-30 min at room temperature. The solution was then added with the unsupplemented medium to a total volume of 3.0 ml and applied to cells which had been washed twice with the unsupplemented medium. The transfected cells were incubated at 37°C in 5% CO_2 for 4 hr. An equal volume of the normal medium without supplementation of antibacterial agents was then added with further incubation for about 20 hr. Cells were washed twice and fed with normal media.

For the CAT assay, a total amount of 5-10 µg of DNA per 60-mm tissue culture dish was used. Cells were cotransfected with the CAT reporter plasmid (pLTR-CAT) and the HBV plasmids or pCEP-*tat* (as a positive control) or a mock plasmid (as a negative control). The ratio of the CAT reporter plasmid and a HBV plasmid used was 4:1. The expression of HBV genes was assayed in HepG2 cells and a total amount of 10 µg of DNA per 60-mm tissue culture dish was used for transfection. To assess transfection efficiency, all transfections were performed in the presence of human growth hormone plasmid pXGH5 (Nichols Institute Diagnostics, San Juan, Capistrano, CA, USA). Secreted human growth hormone was quantitated by radioimmunoassay using a HGH-TGES transient gene expression kit (Nichols Institute Diagnostics). For preliminary detection and normalization of the expression of HBV genes, HBeAg and HBsAg secreted in the cell

media were determined by a Microparticle Enzyme Immunoassay (MEIA) (Abbott Laboratories, Abbott Park, IL, USA).

CAT assay

The CAT assay was modified from the procedure described by Lopata *et. al.*[32] Cell lysates were prepared 48-72 hr after transfection. Cells were washed 3 times with Phosphate-buffered saline solution (PBS) and harvested by incubating with 1 ml of 50 mM Tris-Cl (pH 7.5), 1 mM EDTA, 100-150 mM NaCl at 4°C for 15-20 min. Cells were then scraped off using blue tips, transferred into an Eppendorf tube and centrifuged at 2000 g and 4°C for 1 min to pellet the cells. Cell pellets were resuspended in 100 µl of 0.25 M Tris pH 7.8 and lysed by 3 cycles of freeze and thaw in a methanol-ice bath and 37°C water bath with vigorously vortexing. Cell debris was precipitated by microcentrifugation at 12 000 g for 2-3 min. At this step cell lysates could be stored at -20°C for up to two months for further CAT assay.

For the CAT assay, an optimal amount of cell lysates was used to ensure that the CAT enzyme reaction rate was within the linear range. An aliquot of the cell lysates was mixed with 1M Tris-Cl pH 7.8 to a total volume of 125 µl. Two microliters of 35 mg/ml acetyl Coenzyme A (Sigma Chemical Company, Mississauga, ON, Canada) and 3 µl of 200 GBq/mmol (54 mCi/mmol) D-Threo-[dichloroacetyl-1-^{14}C] Chloramphenicol (Amersham Life Science) were then added. The mixture was incubated at 37°C for 30 min up to 2 hr depending on the quantities of the CAT enzyme expressed which depends on the transfection efficiencies. Typically, the mixture was incubated for 45 min. The reaction mixture was subsequently extracted with 1 ml ethyl acetate. The organic phase was transferred to a fresh Eppendorf tube. The reaction products were concentrated by a Savant SpeedVac Concentrator (Savant Instruments Inc., Farmingdale, NY, USA) and separated by thin-layer chromatography (PE SIL G; Whatman International Ltd., Maidstone, Kent,

England) using $CHCl_3$:Methanol (9:1) as a mobile phase. The converted products were finally analyzed using a phosphoimager (BAS1000, Fuji, Kanagawa, Japan). The relative level of CAT enzyme was determined after normalization for transfection efficiency and total quantity of protein in each cell lysate.

Isolation of extracellular HBV particles

Four to five days after transfection, the culture media from transfected cells were collected and centrifuged in a Sorvall RT6000B Refrigerated Centrifuge at 2500 g for 10 min to remove cellular debris. The extracellular viral particles were pelleted over a 25% sucrose cushion in 50 mM Tris (pH 8.0), 150 mM NaCl and 10 mM EDTA solution using an ultracentrifuge SW 41 rotor at 150 000 g and 4°C for 7-20 hr. The pellets were resuspended in 1 ml of 50 mM Tris (pH 7.5), 150 mM NaCl and 10 mM EDTA. To remove DNA not present in virus particles or contaminating plasmids, 6 mM $MgCl_2$ and 100 µg/ml DNase I were added to the suspension with incubation at 37°C for 30 min. The digested sample was centrifuged at ~12 000 g and 4°C for 10 min. The supernatants were transferred to a fresh Eppendorf tube. The virus particles were precipitated by addition of 300 µl of 26% polyethylene glycol (PEG) 8000, 1.4 M NaCl, and 25 mM EDTA and incubation at 4°C for at least 1 hr. After centrifugation at ~12 000 g and 4°C for 4 min, the pellets were suspended in appropriate solutions. For endogenous polymerase assay, the pellets were suspended in 30 µl of polymerase buffer (50 mM Tris pH 8.0, 40 mM $MgCl_2$, 50 mM NaCl, 1% Nonidet P-40 and 0.3% ß-mercaptoethanol). The pellets were suspended in 50 mM Tris (pH 7.5), 150 mM NaCl and 10 mM EDTA for DNA extraction and southern blot analysis. For detection of complete HBV particles by immunoaffinity capture or immunoprecipitation, the pellets were resuspended in 200 µl of PBS.

Isolation of intracellular HBV core particles

Transfected HepG2 cells in 60-mm tissue culture dishes were lysed by addition of 1 ml of lysis buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 0.25% Nonidet P-40, and 8% sucrose] and incubated for 2-5 min at room temperature. The cell lysate was collected and subjected to microcentrifugation to remove nuclei and cellular debris. To eliminate transfected plasmids and cytoplasmic RNA, the lysate was incubated with 6 mM $MgCl_2$, 100 µg/ml DNase I, and 10 µg/ml RNase A at 37°C for 30 min. The digested sample was centrifuged at ~12 000 g and 4°C for 10 min. The supernatants were transferred to another Eppendorf tube followed by addition of 300 µl of 26% PEG 8000, 1.4 M NaCl, and 25 mM EDTA and further incubation at 4°C for at least 1 hr. The viral core particles were precipitated by microcentrifugation at ~12 000 g and 4°C for 10-15 min. The pellets were then suspended in appropriate solutions as described above.

Endogenous polymerase assay

The assay was modified from the protocols described by Chang *et.al*[19] and Radziwill *et. al.*[17] Viral materials pelleted from culture media or cell lysates were suspended in 30 µl polymerase buffer. Eleven micromole each of dATP, dGTP, and dTTP and 10 µCi of [α-^{32}P] dCTP (3000 Ci/mmol; Dupont, Boston, MA, USA) were added to the mixture. The reaction was performed at 37°C for 1 hr. Chase buffer containing 0.2 mM unlabeled dCTP, and 0.1 mM each of dATP, dGTP, and dTTP was then added with further incubation for 30 min. The reaction was stopped by adding an equal volume of 2X proteinase K buffer [300 mM NaCl, 40 mM EDTA, 20 mM Tris pH 7.5, 2.5% sodium dodecyl sulfate (SDS)]. To release viral DNA from the core particles, proteinase K was added to a final concentration of 1 µg/µl. The reaction mixture was further incubated at 42°C for at least 2 hr. The ^{32}P-labeled viral DNA was isolated by phenol-chloroform extraction and precipitated by incubation with 10 µg of yeast tRNA and 2.5 volumes of

cold ethanol at -20°C overnight. The DNA was pelleted by microcentrifugation at ~12 000 g and 4°C for 10-15 min, resuspended in TE buffer and reprecipitated by incubation with 1/10 volume of 7.5M ammonium acetate and 3 volumes of cold ethanol at -70°C for about 20 min. After removal of the supernatant, the pellets were resuspended in 0.1% SDS in TE buffer. The ^{32}P-labeled viral DNA was analyzed by agarose gel electrophoresis and transferred to a nylon membrane using a standard method for transferring DNA for Southern blot analysis.[33] The relative level of the endogenous polymerase activity was detected by autoradiography and quantitated by a phosphoimager.

Extraction of viral DNA and Southern Blot analysis

Viral materials pelleted from culture media or cell lysates were suspended in 50 mM Tris (pH 7.5), 150 mM NaCl and 10 mM EDTA. Viral DNA was purified by proteinase K digestion and phenol-chloroform extraction, and collected by ethanol precipitation as described in the endogenous polymerase assay. The DNA was finally analyzed by agarose gel electrophoresis. Southern blot analysis was performed using a standard method.[33]

Isolation of total RNA and Northern Blot analysis

Total RNA was harvested from transfected HepG2 cells using TRIzol™ reagent (GIBCO BRL Life Technologies) as described by the manufacturer. Transfected cells grown as monolayers were washed with cold PBS and harvested using 1 ml of cold 0.02% EDTA in PBS. Cells were then transferred into an ice-cold Eppendorf tube and centrifuged to remove the supernatant. The TRIzol™ reagent was added to the cell pellets (1 ml per 5-10 x 10^6 cells). Cells were lysed by pipetting up and down and further incubation at room temperature for 5 min. The viscous solution was then extracted using 0.2 ml of chloroform per 1 ml TRIzol™ reagent used. Total RNA in the aqueous phase was transferred to an Eppendorf tube and precipitated by ice-cold isopropranol (0.5 ml per 1 ml TRIzol™ reagent

used) with incubation at room temperature for 10 min. The supernatant was removed by centrifugation at no more than 12 000 g at 4°C for 15 min. RNA pellets were washed with cold 75% ethanol in diethylpyrocarbonate (DEPC) treated water (≥ 1 ml of 75% ethanol per 1 ml TRIzol™ reagent used). RNA could be stored in 75 % ethanol at -20°C for 1 year or at 4°C for 1 week. The washing was removed by centrifugation at no more than 7500 g at 4°C for 5 min. RNA pellets were finally resuspended in 0.5% SDS in DEPC-treated water

The amount of total RNA was determined by spectrophotometer ($A_{260:280}$ of purified RNA = 1.6-1.8). An equal amount of RNA for each sample was separated on a 1.2% agarose-0.22M formaldehyde gel as described.[34] The RNA blot was prepared and hybridized to ^{32}P-labeled HIV-1 *tat* or HBV probes using standard methods.[33]

Isolation of HBV particles by immunoaffinity-capture

This procedure was based on the screening protocol for antiviral compounds for HBV used by Glaxo Wellcome.[35] The principle of this protocol was to capture HBV virions using anti-HBV surface antigen antibody. The captured virions were then lysed and analyzed by PCR and agarose gel electrophoresis.

Microtiter plate preparation: Each well of a high binding round bottom plate (Corning® 25802) (Corning, Cambridge, MA, USA) was coated with 50 µl of an optimal concentration of a mouse anti-HBV surface antigen monoclonal antibody prepared and purified from H25B10 cells (ATCC No. CRL 8017). The plate was sealed and incubated at 4°C overnight. The antibody solution was then replaced with 100 µl of 0.1% bovine serum albumin (BSA) and 0.02% sodium azide (NaN_3) in PBS and incubated further at 4°C for at least 2 hr.

Immunoaffinity-capture of HBV particles: The coated plate was washed three times with 0.01% Tween 20 and 0.02% NaN_3 in PBS (PBS/T/N) and filled with 10 µl of 0.035% Tween 20 and 0.02% NaN_3 in PBS and 25 µl of each extracellular viral solution

purified from culture media of transfected HepG2 cells as described above. The samples were further incubated at 4°C overnight and washed five times with PBS/T/N and two times with PBS. The solution was pipetted up and down for efficiently washing during each wash.

Isolation and detection of DNA from captured HBV particles: To isolate DNA of the complete HBV particles bound to the antibody, 25 µl of a denaturing solution (0.09 M NaOH and 0.01% Nonidet P-40) was added to each well. The sample was incubated at 37° C for 1 hr and subsequently neutralized with an equal volume of 0.09 M HCl/100 mM Tris (pH 8.3). The sample was stored in an air-tight container at 4°C before further analysis.

The presence of HBV DNA released from the particles was detected by PCR analysis using the upstream primer 5' TCGCTGGATGTGTCTGCGGCGTTTTAT 3' and the downstream primer 5' TAGAGGACAAACGGGCAACATACC 3'. The size of the amplified product would be 114 bp. PCR reaction was performed at a total volume of 25 µl containing 5 µl aliquot of the processed sample as a template, 0.25 µM each of upstream and downstream primers, 6 µM each of dNTPs, 0.01% gelatin, 2 mM $MgCl_2$, 0.125 µl TaqStart antibody (Clontech Laboratories Inc., Palo Alto, CA, USA) and 0.625 unit Taq polymerase (GIBCO BRL Life Technologies) in 1X Taq polymerase buffer (GIBCO BRL Life Technologies). PCR reaction was performed on a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) at the following series of amplification: one cycle of 94°C, 5 min, 30 cycles of 94°C, 30 sec; 55°C, 15 sec; 72°C, 1 min, one cycle of 72°C, 3 min, and holding at 4°C. The DNA product was analyzed by 2% agarose gel electrophoresis.

Immunoprecipitation of HBV particles

The extracellular viral particles harvested from culture media as described above were resuspended in 200 µl of PBS and immunoprecipitated with 1:50 dilution of a goat anti-HBV surface antigen polyclonal antibody (Dako, Carpinteria, CA, USA). The antigen-antibody solution was incubated at 4°C for 2 hr. The solution was further incubated with 100 µl of 10% v/v protein G-sepharose (Pharmacia Biotech) in PBS for 1 hr. The supernatant was removed by microcentrifugation at 200 g at 4°C for 1 min. The viral/protein G-sepharose pellets were washed three times with PBS. The viral DNA was purified by proteinase K treatment and ethanol precipitation and analyzed by Southern blot analysis as described above.

III. Results

Construction of a replicative HBV vector

To generate a replication-competent HBV vector, a foreign gene (the HIV-1 *tat*) was inserted into the unique *Bst*EII site in the tether region in-frame with the P ORF (Figure 2.1). The *tat* insert contained the entire *tat* ORF with its own initiation codon but without a stop codon. This insertion was 39 bp downstream of the preS1 promoter and did not interfere with any other HBV ORFs. A replication-competent form of HBVtat containing two copies of the *Eco*RI-*Eco*RI monomer of the HBVtat recombinant in a head to tail tandem configuration was subsequently constructed (Figure 2.2 a). This dimeric construct was used for studying the functions and characteristics of HBVtat.

Functional expression of HIV-1 *tat* from HBVtat

The expression of the *tat* gene from HBVtat was determined by transfection of HepG2, HeLa and LMH cells. HBVtat was cotransfected with a HIV-1 LTR-CAT reporter plasmid as described in Materials and Methods. The functional activity of the *tat* protein (Tat) was demonstrated by transactivation of the HIV-1 LTR using CAT assay. In HepG2 cells, the basal activity of the CAT enzyme expressed from the HIV-1 LTR-CAT plasmid in the absence of Tat was low (Figure 2.3, lane 2), and increased transactivation activity was observed with the wild type HBV construct compared with the negative control (Figure 2.3, lane 3). However, when HBVtat was present, the HIV-1 LTR was activated to a level similar to that of the Tat positive control (Figure 2.3, lane 4 vs. lane 1). The result illustrated the expression of the Tat function by HBVtat.

The *tat* gene function of HBVtat was also expressed in HeLa and LMH cells but not as well as in HepG2 cells. The transactivation activity of HBVtat in HeLa and LMH cells was about 50% (Figure 2.4) and 45% (Figure 2.5), respectively, of that of the Tat positive

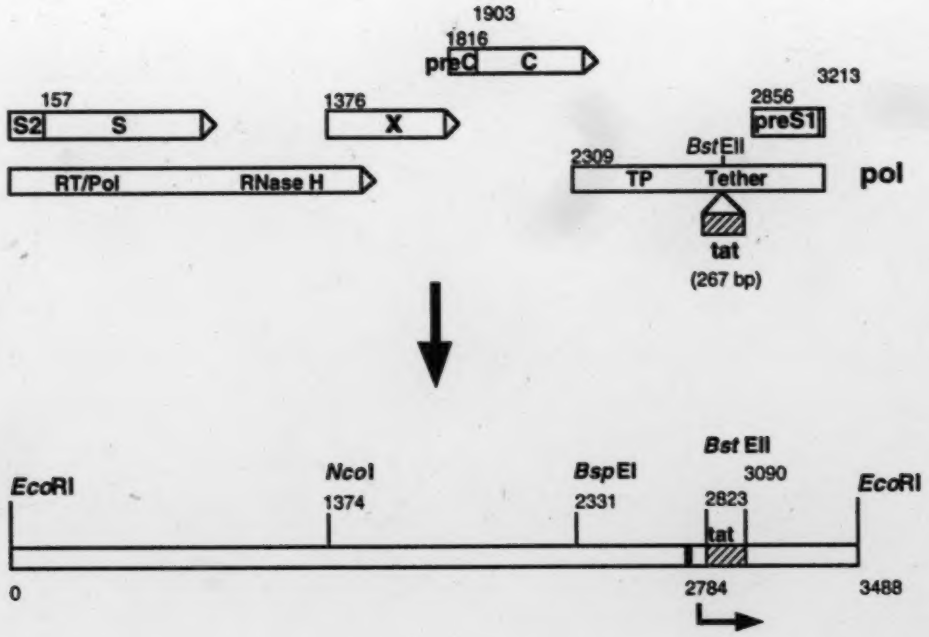
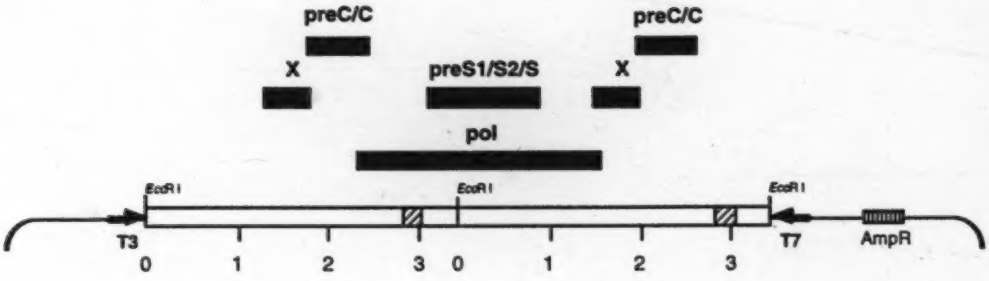


Figure 2.1 Construction of HBVtat. The HIV-1 *tat* gene (267 bp) was inserted into the unique *Bst*EII site in-frame with the P or pol gene and between the promoter (2784 nt) and the initiation codon of the preS1 gene. All the ORFs encoded on the *Eco*RI-*Eco*RI monomer of the HBV genome (3221 bp) are shown with the positions of all initiation codons according to the adw2 subtype. The ORFs start from the blunt end and stop at the arrow end. The four domains of the pol gene corresponding to the functional activities of the Pol protein are indicated. The solid bar is the preS1 promoter and the transcription initiation site of the preS1 RNA (2.4 kb) is indicated by an arrow. The *Nco*I site at the initiation codon of the X gene and the *Bsp*EI site downstream of the initiation codon of the P gene are also shown. RT/Pol, reverse transcriptase and DNA polymerase; TP, terminal protein.

Figure 2.2 Schematic representation of HBV construct and mutants. **a.** Linear map of the HBVtat replication-competent plasmid (pTHBVT-d) (9859 bp) with two *Eco*RI-*Eco*RI monomers in a head to tail tandem configuration subcloned into the pT7T318U vector. All ORFs are depicted by solid bars. The locations of the insertion are indicated by hatched boxes. T3, T3 promoter; T7, T7 promoter; AmpR, ampicillin resistance. **b.** Diagrammatic representation of HBVtat mutations. (i) Site-directed mutagenesis of the X gene at the initiation codon (1376 nt) with an additional stop codon at 1397 nt. (ii) Frameshift mutation in the pol ORF by digestion of the *Bsp*EI site and filling in at 2332 nt to 2336 nt. The mutated or additional nucleotides are shown as boldface letters.

a


preC/C
preC/C
X
preS1/S2/S
X
pol
EcoR I
EcoR I
EcoR I
T3
T7
AmpR
0
1
2
3
0
1
2
3

b

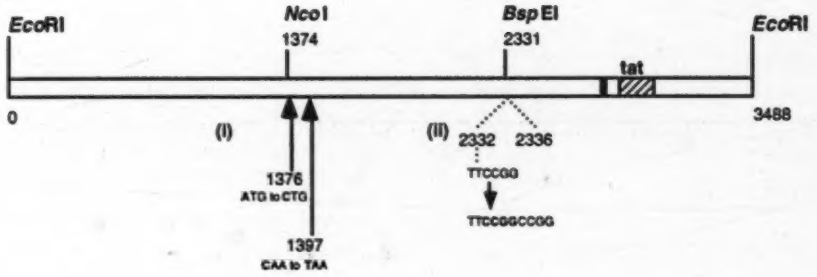
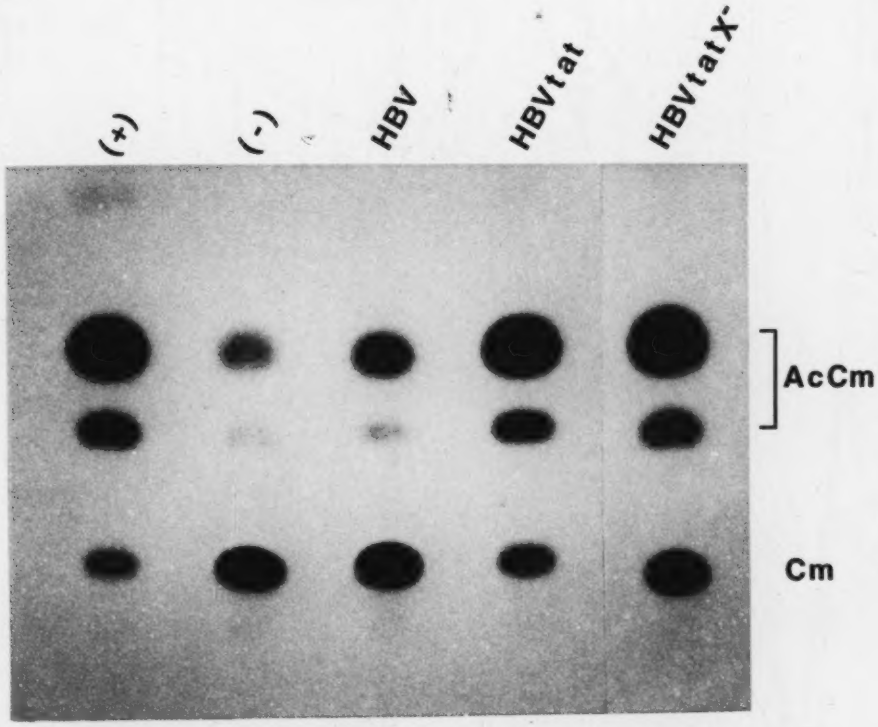
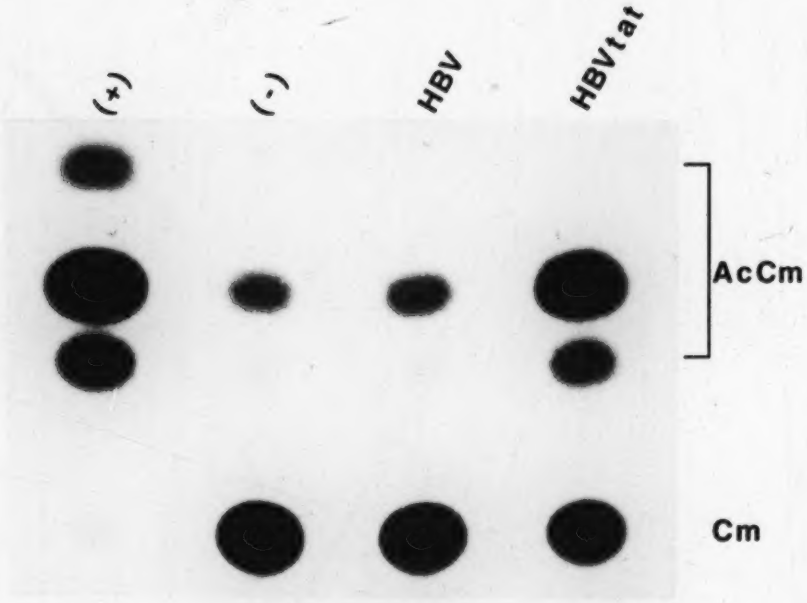

EcoRI
NcoI
1374
BspEI
2331
EcoRI
tat
0
3488
(I)
1376
ATG to CTG
1397
CAA to TAA
(II)
2332
2336
TTCCGG
TTCCGGCCGG

Figure 2.3 Transactivation of HIV-1 LTR by HBVtat expressed in HepG2 cells. HepG2 cells were cotransfected with pLTR-CAT and pCEP-*tat* (lane 1), a mock plasmid (lane 2), wild type HBV (lane 3), HBVtat (lane 4) or X mutant of HBVtat (lane 5). The enzyme activities were determined 48 hr post-transfection. The assay is described in more detail in the text. Relative levels of the CAT expression (normalized to an internal control human growth hormone) are shown as % product converted (AcCm) calculated from three independent experiments with standard deviations . The negative control represents the basal activity of the inactivated HIV-1 LTR. Elevated levels of the CAT enzyme activities reflect transactivation of HIV-1 LTR. AcCm, acetylated products of chloramphenicol; Cm, unacetylated chloramphenicol, a substrate for CAT enzyme.



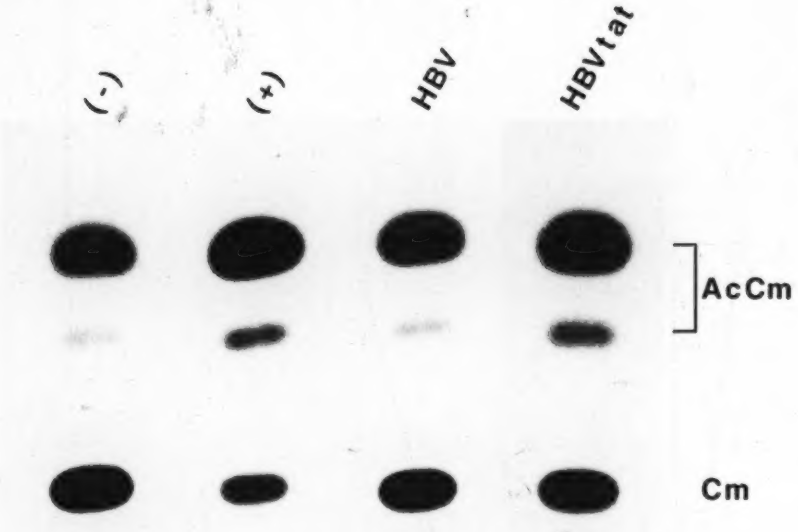
	1	2	3	4	5
Rel. CAT Expression	84.3% ± 1.1	13.6% ± 1.1	28.5% ± 5.8	86.5% ± 1.8	74.1% ± 3.5

Figure 2.4 Transactivation of HIV-1 LTR by HBVtat in HeLa cells. Cells were cotransfected with pLTR-CAT and pCEP-*tat* (lane 1), a mock plasmid (lane 2), wild type HBV (lane 3) or HBVtat (lane 4). Relative levels of the CAT expression were determined 48 hr post-transfection as described in the legend of Figure 2.3.



	1	2	3	4
Rel. CAT Expression	96.6% ± 3.5	9.1% ± 2.6	11.2% ± 2.7	48.1% ± 9.7

Figure 2.5 Transactivation of HIV-1 LTR by HBVtat in LMH cells. Cells were cotransfected with pLTR-CAT and a mock plasmid (lane 1), pCEP-*tat* (lane 2), wild type HBV (lane 3) or HBVtat (lane 4). Relative levels of the CAT expression were determined 48 hr post-transfection as described in the legend of Figure 2.3.



	1	2	3	4
Rel. CAT Expression	20.1% ± 9.7	88.5% ± 0.7	32.6% ± 5.7	42.4% ± 2.8

control. This suggests that HBV is not expressed as efficiently in other cell types as in human liver cells. Further studies of the HBVtat recombinant, therefore, were performed only in HepG2 cells.

Although the wild type HBV construct transactivated HIV-1 LTR to a lesser extent than did the HBVtat recombinant (Figure 2.3, lane 3 vs. lane 4), it was still possible that the transactivation function of HBVtat was enhanced by other HBV genes, such as the X gene.[36] To test this possibility, mutations of the X gene were introduced in HBVtat as shown in Figure 2.2 b. The X mutant (HBVtatX$^-$) retained the transactivation activity, at somewhat reduced levels when compared with the original HBVtat construct (Figure 2.3, lane 5 vs. lane 4).

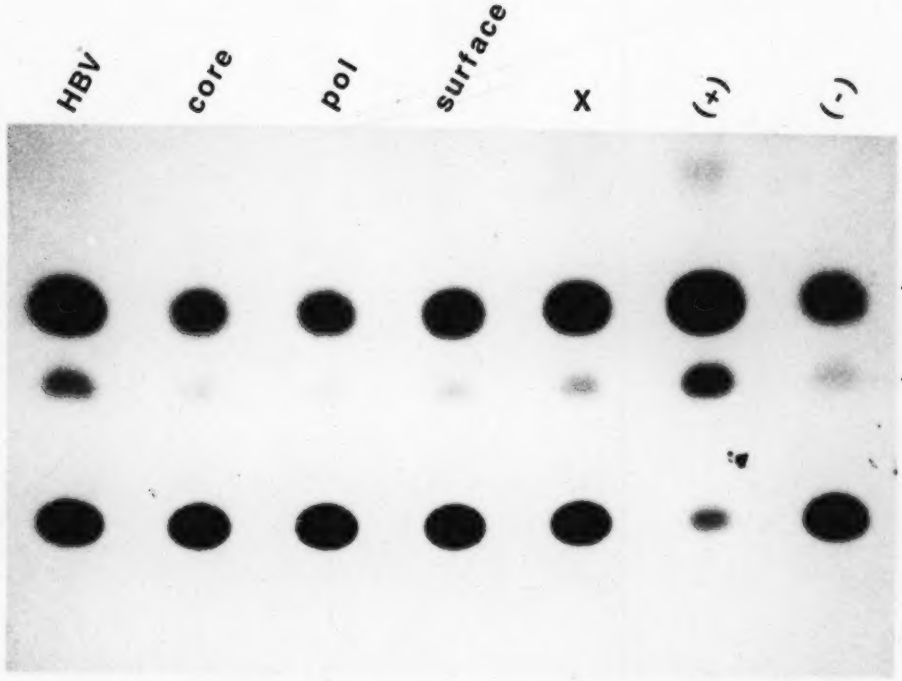
To see if other HBV genes also contributed to the transactivation function, transfection and CAT assays using constructs expressing individual HBV genes were performed in HepG2 cells. The results indicated that the level of the transactivation of the X gene was as high as that of wild type HBV, whereas the transactivation activities of the core, pol or surface gene constructs were insignificant (Figure 2.6).

Taken altogether, these results indicated that the high transactivation activity of HBVtat was due to the *tat* insertion.

Expression of functional Tat independent of a Pol-Tat fusion

Although the *tat* insert was designed to be expressed as a Pol-Tat fusion recombinant using the C promoter, the *tat* ORF was also in optimal proximity to the preS1 promoter (Figure 2.1). It was thus possible that the *tat* gene might be expressed by the preS1 promoter. To determine which promoter was used for the expression of the Tat function, I initially addressed the question by generating a frameshift mutation near the beginning of the P gene in HBVtat (Figure 2.2 b). This mutation disrupted the translation of the pol ORF, thus abolishing the expression of a Pol-Tat fusion protein. Transient expression in HepG2 cells

Figure 2.6 Transactivation of HIV-1 LTR by individual HBV genes. Relative levels of the CAT enzyme activities expressed from HepG2 cells cotransfected with wild type HBV (lane 1), pCHBVC (lane 2), pCHBVP (lane 3), pSV-45 (lane 4), pSG-X (lane 5), pCEP-*tat* (lane 6) or a mock plasmid (lane 7) were determined as described in the legend to Figure 2.3.



	1	2	3	4	5	6	7
Rel. CAT Expression	25.9% ± 3.0	12.4% ± 3.0	14.3% ± 3.8	14.4% ± 2.7	23.7% ± 6.3	89.1% ± 2.8	10.7% ± 1.7

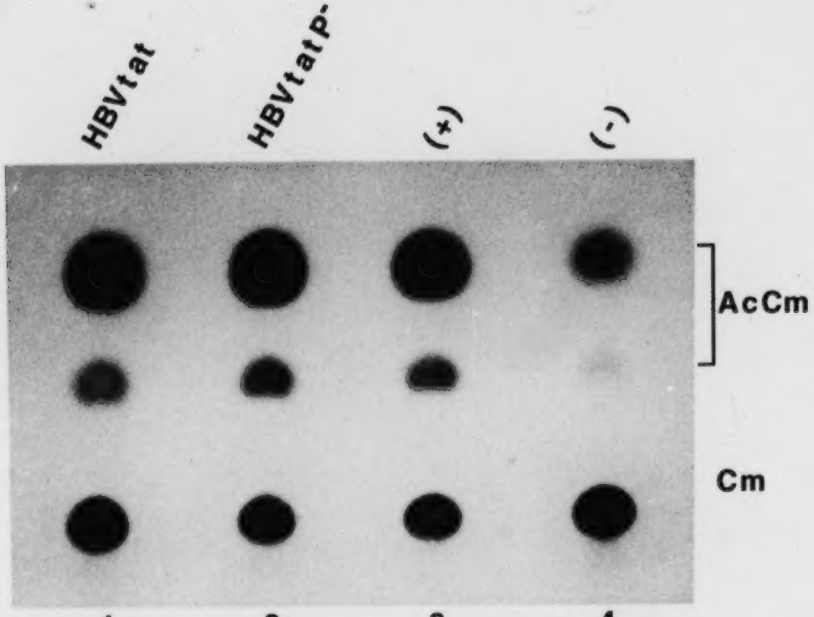
and CAT assay showed that the pol frameshift mutant of HBVtat exhibited a transactivation function similar to that of the original HBVtat construct (Figure 2.7). Thus, although the *tat* gene was in-frame with the P ORF, the transactivation function of HBVtat was not dependent on the expression of Tat as a Pol-Tat fusion protein.

To assess whether the *tat* gene expression could be driven by the preS1 promoter, a Northern Blot analysis was performed. The pregenomic RNA for HBVtat expressed by the C promoter was expected to be about 270 bases longer than that expressed from wild type HBV in accordance with the size of the *tat* insertion. If a *tat* transcript was generated from the preS1 promoter, the size of this subgenomic RNA should also increase by about 270 bases. The sizes of the preS2/S and X messages of HBVtat should be the same as those of wild type HBV. The Northern analysis using an HBV probe detected five species of RNA, 3.70, 3.10, 2.65, 2.05 and 0.80 kb, from HBVtat (Figure 2.8 a, lane 2) and four species of RNA, 3.50, 2.40, 2.10 and 0.80 kb, from wild type HBV (Figure 2.8 a, lane 1). Only three species of RNA transcripts, 3.70, 3.10 and 2.65 kb, were detected from HBVtat by a *tat* probe (Figure 2.8 b, lane 2). The sizes of the pregenomic RNA (3.70 kb) and the subgenomic RNA (2.65 kb) expressed from HBVtat indicated that the *tat* insert was included in transcripts from both the C promoter and the preS1 promoter. It appeared that the *tat* gene sequence was also present in another RNA species of about 3.10 kb in length.

Expression of functional polymerase activity by HBVtat

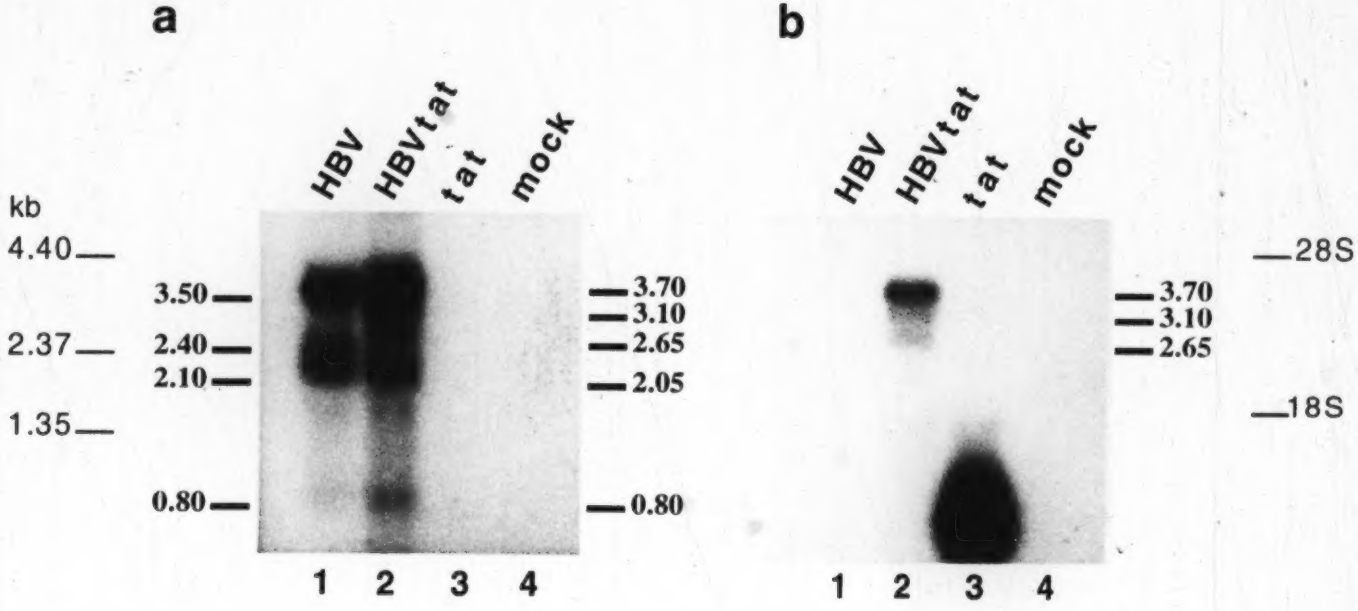
To investigate the effect of the *tat* insertion on expression and function of the pol gene, I performed an endogenous polymerase assay. This assay determines the core-associated viral DNA polymerase activity through incorporation of radioactively-labeled deoxynucleotides into the viral genome. Cytoplasmic lysates and culture media containing intracellular core and extracellular viral particles, respectively, were harvested from HepG2 cells transfected with wild type HBV or HBVtat. The samples were normalized to an

Figure 2.7 Transactivation activity of the P mutant of HBVtat. HBVtat with frameshift mutation in the P gene, HBVtatP$^-$, (lane 2) was determined for transactivation activity in comparison with that of HBVtat (lane 1). Relative levels of the CAT enzyme activities are shown in lanes 3 (positive control) and 4 (negative control).



	1	2	3	4
Rel. CAT Expression	75.0% ± 3.4	85.6% ± 1.4	81.3% ± 10.9	8.8% ± 4.0

Figure 2.8 RNA transcripts expressed from HBVtat detected by Northern Blot analysis. Total RNA from HepG2 cells transfected with wild type HBV (lane 1), HBVtat (lane 2), pCEP-*tat* as a positive control for the *tat* gene expression (lane 3) and a mock plasmid (lane 4) were separated on a 1.2% agarose-0.22M formaldehyde gel. The RNA was transferred to Hybond-N membrane (Amersham Life Science) and hybridized with a ^{32}P-HBV DNA probe. The same blot was stripped by washing in a boiling 0.5% SDS solution as described by the membrane manufacturer and rehybridized with a ^{32}P-*tat* DNA probe. **a.** Autoradiograph of Northern blot analysis using a ^{32}P-HBV DNA probe. Sizes of the transcripts expressed from the cells transfected with wild type HBV and HBVtat that contain the HBV sequences are shown on the left and right, respectively. **b.** Autoradiograph of Northern Blot analysis using a ^{32}P-*tat* DNA probe. Sizes of the transcripts expressed from the cells transfected with HBVtat are shown to the right. Positions of RNA size markers and 28S and 18S rRNA are shown at left and right, respectively.


a
HBV
HBVtat
tat
mock
kb
4.40
2.37
1.35
3.50
2.40
2.10
0.80
3.70
3.10
2.65
2.05
0.80
1 2 3 4
b
HBV
HBVtat
tat
mock
3.70
3.10
2.65
28S
18S
1 2 3 4

internal transfection control (secreted human growth hormone) and to the amounts of hepatitis B e antigen (HBeAg) and hepatitis B surface antigens (HBsAg) secreted into the culture media. The reaction products were analyzed by agarose gel electrophoresis and autoradiography. Labeled DNA bands, corresponding to relaxed circular and linear double stranded DNA genome and single stranded DNA, were detected, albeit at reduced levels, as a result of the DNA polymerase activity of HBVtat (Figure 2.9 a and b, lanes 1), indicating that the Pol-Tat fusion of HBVtat retained the polymerase function.

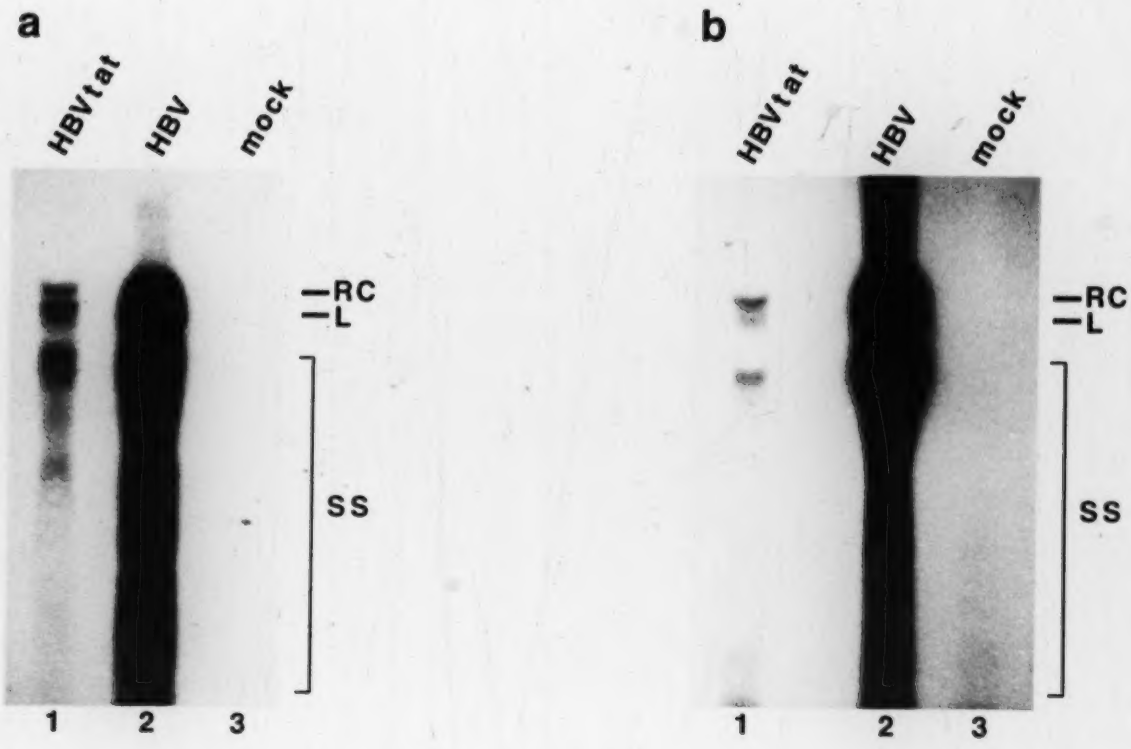
Compared with wild type HBV, levels of endogenous polymerase activities in the intracellular core particles of HBVtat measured by a phosphoimager were about 4%, and in the extracellular viral particles of HBVtat were about 1.5%. Southern Blot analysis of the intracellular core and extracellular viral particles of HBVtat confirmed these results. Thus, the insertion of the 267 bp *tat* gene within the tether region of the pol gene reduced but did not abolish the polymerase function.

Synthesis of complete viral particles by HBVtat

The ability to form complete viral particles would be important for the use of recombinant HBV as a vector in gene transfer. A therapeutic gene carried in the viral genome would be efficiently and specifically introduced to target cells via infection. I thus determined whether intact viral particles could be produced by HBVtat.

Previous studies have established that the L protein is absolutely required for the formation and secretion of HBV free virus particles.[37,38] The insertion of the *tat* gene between the initiation codon and the promoter of preS1 gene might interrupt the expression of the L protein and, therefore, would affect production and secretion of viral particles. The detection of endogenous polymerase activity in extracellular products of HBVtat-transfected HepG2 cells suggested that complete virus particles carrying the HBVtat

Figure 2.9 Endogenous polymerase activities of HBVtat. The viral core particles and cell-free particles were isolated from the cytoplasmic lysates and culture media of transfected HepG2, respectively, 4-5 days post-transfection. Core particles and extracellular viral particles produced from approximately equal amounts of transfected cells were used in each assay after normalizing to an internal control for transfection efficiency (hGH). Endogenous polymerase activities were determined as described in the text. **a.** Endogenous polymerase activities in intracellular core particles. **b.** Endogenous polymerase activities in extracellular viral particles or cell-free particles. RC, relaxed circular; L, linear; SS, single stranded.


a
HBVtat
HBV
mock
RC
L
SS
1
2
3
b
HBVtat
HBV
mock
RC
L
SS
1
2
3

recombinant genome had been synthesized. To confirm this, I carried out an immunoaffinity-capture assay to directly analyze the secreted HBV particles.

Extracellular virus particles from transfected HepG2 cells were captured using anti-HBV surface antibody as described in Materials and Methods, and PCR was performed to detect the HBV genomic DNA. To eliminate contamination of plasmid DNA carried over from transfections, the samples were treated with DNase I prior to the immunoaffinity capturing. Cells transfected with a nonreplicative HBV construct (pTHBVP$^-$) were included as controls, treated or untreated with DNase I. No viral DNA could be detected in samples transfected with pTHBVP$^-$ if treated with DNase I, whereas plasmid DNA contaminants were detected if the samples were not treated with DNase I (Figure 2.10, lanes 3 and 4). Thus, contamination of transfecting plasmids was eliminated by the DNase I treatment. HBV genomic DNA was detected in DNase I treated culture media from cells transfected with wild type HBV or HBVtat (Figure 2.10, lanes 1 and 2) but not from those transfected with the nonreplicative HBV plasmid or a mock plasmid (Figure 2.10, lanes 3 and 5).

The synthesis of the HBVtat viral particles was also demonstrated by immunoprecipitation and Southern Blot analysis (Figure 2.11). HBeAg and HBsAg produced by HBVtat were also examined, both of which were detected at levels similar to those of wild type HBV (Table 2.1).

It was concluded from these studies that complete viral particles were synthesized by HBVtat.

Figure 2.10 Immunoaffinity-capture assay to detect complete viral particles produced from HBVtat. The extracellular products were captured from culture media of cells transfected with wild type HBV (lane 1), HBVtat (lane 2), nonreplicative HBV (lane 3 and 4) and mock (lane 5) as described in Materials and Methods. DNA of the captured HBV or HBVtat particles was determined by PCR and analyzed by 2% agarose gel electrophoresis. The actual amplified product was 114 bp in size. The DNA band detected in the sample of nonreplicative HBV without DNase I treatment (lane 4) was a contamination of the transfecting plasmid. Marker, 1 kb DNA ladder (GIBCO BRL, Life Technologies); +, with DNase I treatment; -, without DNase I treatment.

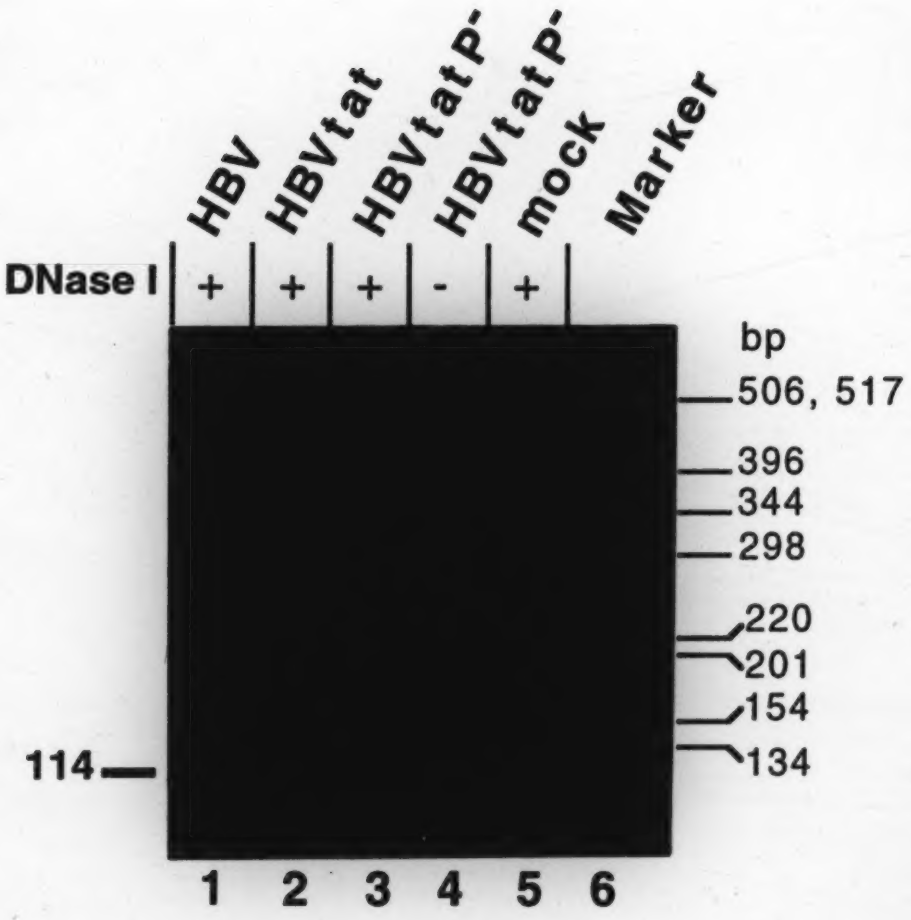

HBV
HBVtat
HBVtatP-
HBVtatP-
mock
Marker
DNase I
+
+
+
-
+
bp
506, 517
396
344
298
220
201
154
134
114
1
2
3
4
5
6

Figure 2.11 Immunoprecipitation and Southern Blot analysis to detect complete viral particles produced from HBVtat. The extracellular products of cells transfected with HBVtat (lane 1), wild type HBV (lane 2) and mock (lane 3) were immunoprecipitated as described in Materials and Methods. The viral DNA was detected by Southern Blot analysis using a ^{32}P-HBV DNA probe.

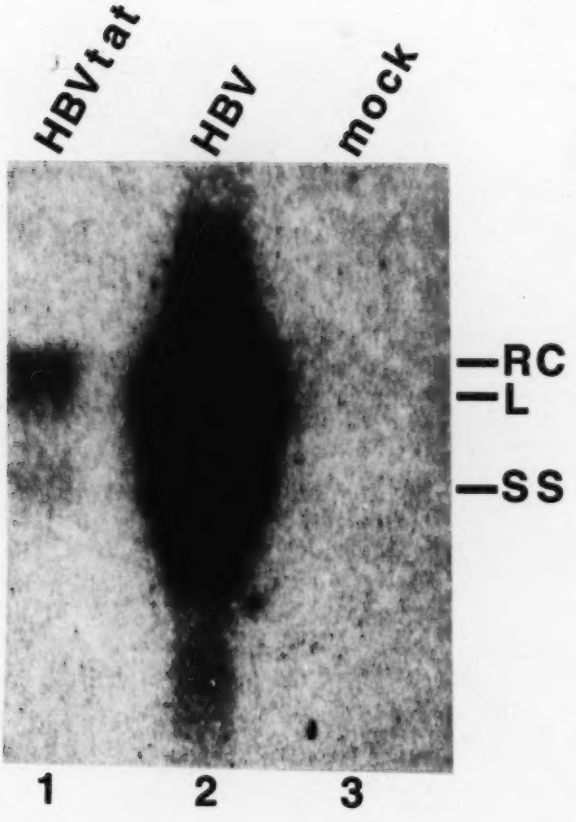

HBVtat
HBV
mock
RC
L
SS
1
2
3

Table 2.1 Detection of HBsAg and HBeAg produced by HBVtat

Samples[a]	Amounts (S/N[b] ± standard deviation)	
	HBsAg	HBeAg
HBV	56.03 ± 27.35	249.18 ± 65.29
HBVtat	60.06 ± 13.83	226.56 ± 85.76
mock	1.20 ± 0.04	1.18 ± 0.05

[a] Samples were culture media of HepG2 cells transfected with wild type HBV, HBVtat or mock and were assayed for HBsAg and HBeAg by MEIA.

[b] HBsAg and HBeAg produced were determined as S/N values as described by the manufacturer. ≥ 2.00 S/N is the cut off rate for positive results. According to the manufacturer, ≥ 7.00 S/N of HBsAg detected is equivalent to 4-15 ng/ml concentration but the absolute concentration of HBeAg is not determined.

IV. Discussion

Gene therapy has become one of the most attractive therapeutic strategies and, if applicable, can treat both acquired and genetic disorders. To be successful, however, gene therapy requires efficient tools for delivery and targeting, and the genes need to be expressed at therapeutic levels. For these reasons, viruses have been used as the best gene delivery mediators.

A number of inherited or metabolic disorders affect the liver. Patients with liver dysfunction often endure a short life span. Although liver transplantation has been successfully performed to save lives of patients with severe liver diseases, the transplanted patients still require long term immunosuppressive treatment. Moreover, transplantation is also limited by the availability of liver donors and the procedure carries significant morbidity and mortality risk. Gene therapy, therefore, is a promising approach for correcting genetic liver defects. Owing to its natural hepatotropism, hepadnaviruses could be the most effective tools for liver gene transfer and may overcome the problems often associated with other liver-directed gene transfer systems.[39]

This article exploits the potential of using HBV as a gene delivery vector. In this study, I have demonstrated that a foreign gene, the HIV-1 *tat*, can be expressed and fully functional in the context of the full length HBV genome. Expression of the *tat* gene seems to be specific to human liver cells as it exhibits full transactivation function in HepG2 cells. Diminished expression of the *tat* gene controlled by the endogenous HBV promoter/enhancer elements in HeLa and LMH cells reflects the high tissue and species specificity of hepadnaviruses.[18,40-42] DHBV also replicates more efficiently in chicken hepatoma cells (LMH) than in human liver cells (Huh-7 and HepG2).[43] This shows the necessity of using HBV as a vector in hepatic gene transfer although it may be advantageous to use nonhuman pathogenic hepadnaviruses as gene transfer tools.

The Northern blot analysis suggests that the *tat* gene inserted in the tether region is transcribed by both the C promoter and preS1 promoter. Since the Pol-Tat fusion expressed by the C promoter is not accountable for the transactivation activity of HBVtat, the functional Tat is most likely expressed as a Tat-Pol fusion product using the *tat* initiation codon. It is known that Tat functions in the nucleus and HBV Pol interacts with the 5' epsilon sequence of the pregenomic RNA and is encapsidated into core particles in the cytoplasm.[44,45] It is conceivable that the Pol-Tat fusion is encapsidated into the viral core particles in the cytoplasm and thus is not transported into the nucleus where the Tat protein would function. Since the entire Pol protein is required for encapsidation and packaging of the cytoplasmic viral core particles,[25,26] the Tat-Pol fusion recombinant lacking the N-terminal domain of Pol would not be incorporated into the core particles. Therefore, the Tat-Pol fusion may migrate to the nucleus and confer transactivation activity. Multiple attempts for detecting the Tat-Pol fusion by Western blot analysis, immunoprecipitation and immunofluorescence staining have not been successful possibly due to the low levels of protein expression or the lack of optimal anti-Tat antisera.

Although the Tat-Pol fusion was most likely synthesized from transcripts of the preS1 promoter, I cannot officially exclude the possibility of its synthesis from the pregenomic RNA by internal initiation, a mechanism used for the synthesis of HBV Pol.[46]

An additional 3.1 kb RNA containing the *tat* sequence was detected in HBVtat but not in wild type HBV transfected cells. The near-genomic size suggests that this *tat* RNA species might originate from the pregenomic RNA. RNA splicing has been reported in hepadnaviruses.[47,48] Sequence analysis of HBVtat has revealed consensus splice donor sites on the HBV genome flanking the *tat* insert and a consensus splice acceptor site and a branch point within the *tat* sequence. Thus, I speculate that this 3.1 kb transcript is a spliced product of the pregenomic RNA of HBVtat.

Insertion of the *tat* gene in-frame with the pol gene has reduced the endogenous polymerase activity of the Pol protein. It is possible that the insertion interferes with the

structural conformation of the Pol protein. Nevertheless, this study shows that the HBVtat recombinant is replicative and complete viral particles are formed. Analyses of the amounts of HBsAg and HBeAg produced from HBVtat suggest that the expression of these HBV structural genes is not significantly affected by the *tat* insertion. One major concern about the design of HBVtat was that the *tat* insertion might interfere with the L protein synthesis which has been shown to be absolutely necessary for virion assembly[37,38] and binding to cellular receptor(s).[49,50] The detection of extracellular viral particles indicates that the L protein is made and particles are fully assembled. Therefore, it is highly possible that the recombinant viruses could infect liver cells.

Although attempts to produce defective and recombinant hepadnavirus particles have been reported,[51] this is the first study clearly demonstrating expression of a foreign gene in the context of a replication-competent HBV genome. Recombinant viral particles are produced, even though the infectivity or transduction ability of the recombinant viruses remains to be demonstrated. The majority of viral vectors developed for gene delivery are replication-defective; as a result, high titers of the recombinant viral vectors are usually required for efficient gene transduction. Since this HBV recombinant is replication-competent, a low titer of the recombinant viral vector may be sufficient for effective gene transfer.

An HBV vector would naturally be the most appropriate tool for liver gene transfer. Due to its ability to infect non-dividing hepatocytes, transduction rates of HBV vectors can be very efficient, particularly in comparison to Moloney murine leukemia virus-derived retroviral vectors which require cell division for gene transfer. The fact that a large number of HBV infected humans appear to continuously harbor the HBV genome and express HBsAg without pathogenic consequences offers a great advantage in term of long-term maintenance and expression of a recombinant HBV vector bearing a desired gene. A recombinant HBV genome might be attenuated to the extent that the HBV-associated pathogenesis is diminished.

This study has clearly demonstrated the feasibility of constructing such a replicative HBV vector. Further development and studies of HBV as therapeutic gene transfer vectors are necessary.

V. Bibliography

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CHAPTER 3*

Expression of Zeocin™ resistant gene (*Sh ble* gene) by hepatitis B virus replicative and nonreplicative vectors

I. Introduction

Novel approaches of gene transfer targeted to the liver *in vivo* could revolutionize the treatment of many acquired and genetic liver diseases, particularly those, such as phenyketonuria,[1] for which a satisfactory treatment is not available. Targeting *in vivo* could be preferable to *ex vivo* liver gene therapy since hepatocyte isolation and reimplantation are not required. For high efficacy and safety, however, an *in vivo* strategy requires a suitable delivery system to mediate the expression of transgenes, specifically and optimally, at therapeutic levels in the liver.

In vivo liver-directed gene therapy using viral vectors, especially retroviral and adenoviral vectors, has been studied extensively; however, problems related to the use of these systems have been described.[2-5] An important drawback often associated with the use of such vectors results from the wide range of tissues infected by these viruses. The consequences of nonspecific expression in nonhepatic tissues remain largely unknown. Therefore, liver-specific gene delivery vectors or regulatory elements have been investigated intensively in order to circumvent this problem. An initial *in vitro* study on the strength of different promoters in primary hepatocytes has been reported.[6] Many attempts have been made to construct retroviral or adenoviral vectors carrying liver-specific promoter/enhancer elements for directly targeting gene expression to the liver. The cellular

*The data of Figure 3.7 presented in this chapter has been published in Gene Therapy (1997) 4: 1330-1340 by S. Chaisomchit, D. L. J Tyrrell and L.-J. Chang. Most of the data presented in this chapter is submitted for publication in Gene Therapy,1998.

liver-specific promoters including human alpha 1-antitrypsin (hAAT), murine albumin, rat phosphoenolpyruvate carboxykinase and rat liver fatty acid binding protein promoters have been evaluated for specificity and the extent of gene regulation in retroviral vectors in hepatocytes *in vitro* and *in vivo*.[7] The hAAT promoter exhibits an efficient and stable level of expression of transgenes in recombinant retroviral vectors in hepatocytes.[7,8] The addition of the liver-specific apolipoprotein E enhancer upstream of the hAAT promoter in a retroviral vector seems to increase the expression of hAAT dramatically.[9] The relative strength of a series of viral and cellular promoter/enhancer units, such as Moloney murine leukemia virus long terminal repeat (LTR), Rous sarcoma virus LTR, human cytomegalovirus immediate early gene promoter, human elongation factor 1α (EF1α) promoter, murine albumin enhancer/promoter and murine transthyretin enhancer/promoter, have been evaluated for hepatic gene expression *in vitro* and *in vivo* using adenoviral vectors as gene mediators.[10] Of the promoters tested, the human EF1α promoter exhibits the most efficient and persistent function in the liver. In another example, Connelly *et al*[11] showed that inclusion of the liver-specific murine albumin promoter in an adenoviral vector restricted the expression of human blood coagulation factors VIII in the liver.

Due to its natural hepatotropism, which restricts both viral replication and gene expression to liver cells,[12-14] hepadnaviruses represent an attractive gene expression system for hepatocyte-directed gene transfer. Loser *et al*[14] showed by *in vitro* studies that the hepatitis B virus (HBV)-derived promoter/enhancer elements, including the C promoter, C promoter linked with enhancer I, preS1 promoter and HBV enhancer II-cytomegalovirus (CMV) hybrid promoter, exhibit a high specificity in liver-derived cells. A further study demonstrates that the HBV-CMV hybrid promoter/enhancer elements have strong liver-specificity *in vivo* in controlling the expression of the LDL receptor using adenoviral vectors.[15]

Our laboratory has been interested in developing HBV as a gene delivery vector. Previously, we described successful construction of a replicative HBV vector for

expressing the human immunodeficiency virus type 1 (HIV-1) *tat* gene in-frame with the HBV polymerase protein (Pol).[16] Expression of the *tat* gene has been studied in liver- and nonliver-derived cells, including human hepatoma (HepG2), chicken hepatoma (LMH) and human cervical carcinoma (HeLa) cells, and appears to be regulated by the C promoter and/or the preS1 promoter. The levels of expression in human hepatoma cells are higher than those in nonhepatic-origin cells.

In this study, further investigations were performed to determine whether a replicative HBV vector can accommodate a gene larger than HIV-1 *tat*. The Zeocin™ resistant gene (Zeo^R) or *Sh ble* gene was chosen for this study. This gene, originally cloned from *Streptoalloteichus hindustanus*,[17] encodes a 13.6 kD protein that can inhibit the activity of the Zeocin™ antibiotic. The Zeocin™ antibiotic is a basic, water soluble copper-chelated glycoprotein isolated from *Streptomyces verticillus* and is a member of the phleomycin family of antibiotics.[18] This antibiotic exhibits toxicity towards a broad range of prokaryotic and eukaryotic cells. It is activated upon removal of the copper, when the antibiotic enters cells, and will bind the DNA, thus causing DNA cleavage and subsequent cell death.[18] The presence of the Zeo^R protein inhibits the antibiotic activity via the binding in a stoichiometric manner, therefore preventing cellular DNA cleavage.[19]

The possibility of constructing a nonreplicative HBV vector was also investigated in this study since, in fact, a nonreplicative viral vector may be more favorable for a therapeutic use. Moreover, because HBV is very small and compact,[20,21] the insertion of a large gene may interfere with its replication. In this chapter, the expression of Zeo^R from both replicative and nonreplicative HBV vectors as well as the replication capability of both recombinant vectors will be described.

II. Materials and Methods

Plasmid construction

To construct a replicative HBVZeo recombinant, the Zeo^R or *Sh ble* gene (*Streptoalloteichus hindustanus* bleomycin gene) was subcloned without a stop condon from pcDNA3.1/Zeo (Invitrogen, San Diego, CA, USA) into the unique *Bst*EII site in the HBV polymerase ORF (P) of pTHBV.[16] The subcloning was performed by PCR as described in Chapter 2 using the upstream primer 5' TGCGGGTCACCAATGGCCAAG-TTGACCAGTGCC 3' and the downstream primer 5' ATATGGTGACCCGTCCTGCTC-CTCGGCCACGAAGTG 3' (the *Bst*EII sites are underlined). DNA sequencing confirmed the actual sequence. A replication-competent form (pTHBVZeo-d) which was used for the study contained two copies of a head to tail configuration of HBVZeo monomer.

A nonreplicative HBVZeoS recombinant was also constructed by subcloning the entire Zeo^R ORF with a stop codon from pcDNA3.1/Zeo (Invitrogen) into the unique *Bst*EII site in the P ORF of pTHBV[16] by PCR using the upstream primer 5' TGCGGGTCACCAAT-GGCCAAGTTGACCAGTGCC 3' and the downstream primer 5' ATATGGTGACCC-TCAGTCCTGCTCCTCGGCCACGAAGTG 3' (the *Bst*EII sites are underlined). DNA sequencing confirmed the actual sequence. Since the insertion had a stop codon and was in-frame with the P gene, the expression of Pol was eliminated. A dimeric form of HBVZeoS (pTHBVZeoS-d) was constructed by ligation, head to tail, of two copies of the full length HBVZeoS monomer in the pT7T318U vector (Pharmacia Biotech, Uppsala, Sweden) and used for study of replication of HBVZeoS.

pCHBVP used in the endogenous polymerase assay with Pol complementation was constructed as described.[16]

Cell culture and transfection

HepG2 cells were cultured and maintained at 37°C in 5% CO_2 in Auto-Pow MEM Eagle (modified) medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 10 mM sodium bicarbonate, 2 mM L-glutamate, 10% fetal bovine serum, 50 units/ml penicillin G sodium, 0.01 mg/ml streptomycin and 50 units/ml nystatin.

Transfections were performed using Lipofectin (GIBCO BRL Life Technologies, Gaithersburg, MD, USA) as described in Chapter 2. A total amount of 10 µg of DNA per 60-mm tissue culture dish was used for transfection. Endogenous polymerase assays with Pol complementation were performed by cotransfection of equimolar quantities of the HBV plasmids and the HBV polymerase expressing plasmid (pCHBVP) into HepG2 cells. To assess transfection efficiency, all transfections were performed in the presence of human growth hormone plasmid pXGH5 (Nichols Institute Diagnostics, San Juan, Capistrano, CA, USA). Secreted human growth hormone was quantified by radioimmunoassay using a HGH-TGES transient gene expression kit (Nichols Institute Diagnostics). The quantities of the HBV e antigen (HBeAg) and surface antigens (HBsAg) secreted into the cell media were determined by a Microparticle Enzyme Immunoassay (MEIA) (Abbott Laboratories, Abbott Park, IL, USA).

Determination of cell viability by trypan blue exclusion

Adhering cells were harvested by trypsinization and transferred to a 15-ml tube and centrifuged in a Sorvall RT6000B Refrigerated Centrifuge (Dupont, Mississauga, ON, Canada) at 1500 g at 4°C for 3 min. Cells were resuspended in 100-500 µl of culture medium depending on the number of cells. An equal volume of 0.1% w/v trypan blue in PBS was added to an aliquot of cells. The mixture was incubated at room temperature for 2-3 min to allow dye uptake. Cells were then loaded into a hemocytometer and scored for

viable cells (blue cells = dead cells). Typically, a minimum of 200 total cells per four separate fields were counted. The percentage of viable cells was calculated as follows:

$$\% \text{ Viable cells} = \frac{\text{Number of cells excluding dye}}{\text{Total number of cells plated}} \times 100$$

Zeocin™ sensitivity test in HepG2

Cells were grown in 60-mm tissue culture dishes for about 48 hr to 90-100% confluent (2.5-3.0 $x10^6$ cells per dish). The cells were trypsinized, transferred to 100-mm tissue culture dishes and fed with media containing varying concentrations of Zeocin™ (100, 250, 500 and 750 µg/ml). The cells were incubated at 37°C in 5% CO_2 and replenished with the selective medium every 2 days. The percentages of surviving cells over time were determined by trypan blue exclusion as described above and counting in a hemocytometer.

Cell viability test for Zeo^R expression

Two days after transfection, cells were trypsinized and transferred to a 100-mm tissue culture dish. Cells were fed with media containing 250 µg/ml Zeocin™ and incubated at 37°C in 5% CO_2. Media were changed every 2-3 days. Before adding new media, each sample was washed once to remove floating dead cells. After fourteen days of growing in the Zeocin™ containing media, cells were harvested by trypsinization. Viable cells were evaluated by the trypan blue exclusion and quantified using a hemocytometer as described above.

Isolation of extracellular HBV particles

Four to five days after transfection, the culture media from transfected cells were collected and centrifuged in a Sorvall RT6000B Refrigerated Centrifuge at 2500 g at 4°C for 10 min to remove cellular debris. The extracellular viral particles were isolated by

ultracentrifugation over a 25% sucrose cushion as described in Chapter 2. For an endogenous polymerase assay, the pellets were suspended in 30 µl of polymerase buffer (50 mM Tris pH 8.0, 40 mM $MgCl_2$, 50 mM NaCl, 1% Nonidet P-40 and 0.3% ß-mercaptoethanol).

Endogenous polymerase assay

Viral materials pelleted from culture media or cell lysates were suspended in 30 µl polymerase buffer. Endogenous polymerase assays were performed as described in Chapter 2. The final products (^{32}P-labeled viral DNA) were purified by phenol-chloroform extraction and ethanol precipitation and analyzed by agarose gel electrophoresis. The DNA was transferred to a nylon membrane using a standard method.[22] The relative level of the endogenous polymerase activity was detected by autoradiography and quantified by a phosphoimager (BAS1000, Fuji, Kanagawa, Japan).

III. Results

Rationale and experimental design

The objectives of this part of the study were to determine whether a large gene, i.e. larger than the HIV-1 *tat*, could be expressed by a HBV replicative vector and whether that recombinant HBV construct was still replication-competent. The Zeo^R gene was chosen for the study since its size, about 100 base pairs (bp) larger than the HIV-1 *tat*, was suitable for the purpose. A much larger reporter gene might, theoretically, totally eliminate the replication and packaging of the HBV recombinant. The results of this study should provide an idea of the size limit of a foreign gene that could be accommodated by the HBV vector such that retained its replication capability. The Zeo^R gene (381 bp) was inserted in-frame with the HBV P gene. This recombinant was designated as HBVZeo (Figure 3.1).

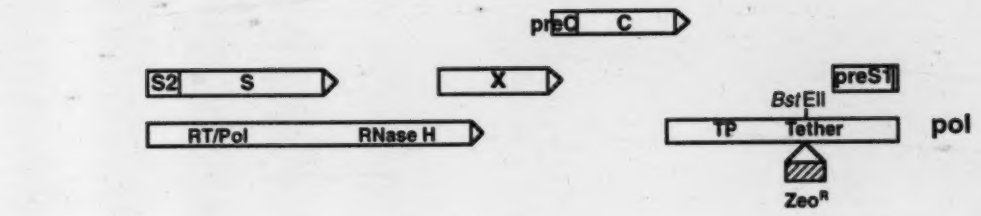
The possibility of construction and expression of the Zeo^R gene by a HBV nonreplicative vector was also investigated. An HBVZeoS nonreplicative recombinant was constructed by insertion of the Zeo^R gene with a stop codon (384 bp) in-frame with the P gene which rendered this construct replicative-incompetent (Figure 3.1).

The dimeric constructs of HBVZeo and HBVZeoS were used for studying gene expression and replication capability of the HBV replication-competent and replication-incompetent vectors, respectively. The expression of the Zeo^R gene was determined by transfection of HepG2 cells and quantitation of cells resistant to the Zeocin™ antibiotic.

Zeocin™ sensitivity of HepG2

Prior to the study of expression of HBVZeo and HBVZeoS, HepG2 cells were tested for sensitivity to Zeocin™ in order to establish the optimal concentration required to kill the parental HepG2 cells and to select the transfectants. The cells were incubated with

Figure 3.1 Schematic representation showing monomers of HBV recombinant vectors. The Zeo^R gene was inserted into the unique *Bst*EII site in-frame with the P gene and between the promoter (at the 2784 nucleotide) and the initiation codon of the preS1 gene. Replication-competent HBVZeo and replication-incompetent HBVZeoS contained the Zeo^R insert without and with a stop codon, respectively. All the ORFs encoded on the *Eco*RI-*Eco*RI monomer of the HBV genome (3221 bp) with the positions of all initiation codons according to the adw2 subtype are shown. The ORFs start from the blunt end and stop at the arrow end. The four domains of the P (pol) gene corresponding to the functional activities of the Pol protein are indicated. The hatched box represents the Zeo^R insert. The asterisk indicates a stop codon of the Zeo^R gene in HBVZeoS. The solid bar and the arrow indicate the promoter and transcription initiation site of the preS1 gene, respectively. RT/Pol, reverse transcriptase and DNA polymerase; TP, terminal protein.


preC
C
S2
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RT/Pol
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Bst EII
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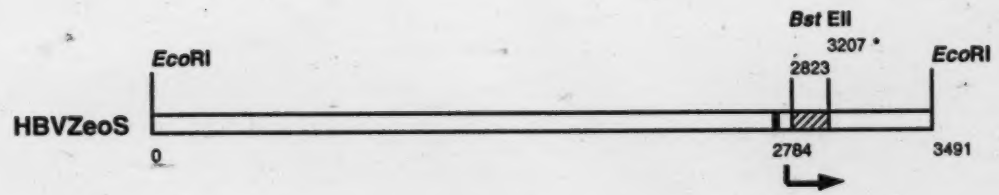
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Zeocin™ at various concentrations as described in Materials and Methods and the percentages of cell viability were evaluated for a period of about 14 days. The study showed that growth of HepG2 cells was inhibited at Zeocin™ concentrations of 250 µg/ml or above (Figure 3.2).

The growth of HepG2 cells transfected with pcDNA3.1/Zeo (a positive control) in media containing 500 and 750 µg/ml Zeocin™ was tested. It was found that these concentrations seemed to be too high for the selection of the Zeo^R gene expression since the number of cells transfected with this Zeo^R expressing plasmid that survived in these concentrations was very low (not significantly different from the negative controls) after 14 days of incubation. Thus, these high concentrations were not suitable for selection for the Zeo^R expression. A Zeocin™ concentration of 250 µg/ml, therefore, was optimal for use in further studies of the Zeo^R expression in HepG2 cells. Significant quantities of cells transfected with pcDNA3.1/Zeo grew in the presence of 250 µg/ml Zeocin™ (as also evident in Figures 3.3, 3.4 and 3.6).

Expression of Zeo^R and polymerase activity of HBVZeo

Expression of Zeo^R and HBV genes from HBVZeo was studied by transfection of HepG2 cells. Cells expressing the Zeo^R protein were quantified as the percentage of cells remaining viable on incubation in media containing 250 µg/ml Zeocin™. During the incubation period, the viability rate of cells transfected with HBVZeo decreased at about the same rate as that of the negative control (Figure 3.3). After 14 days incubation in the selective media, cells transfected with HBV (a negative control), HBVZeo and pcDNA3.1/Zeo (a positive control) survived at average rates of 2.7%, 2.4% and 49.5%, respectively (Figure 3.4). I concluded that no functional Zeo^R protein was expressed from HBVZeo.

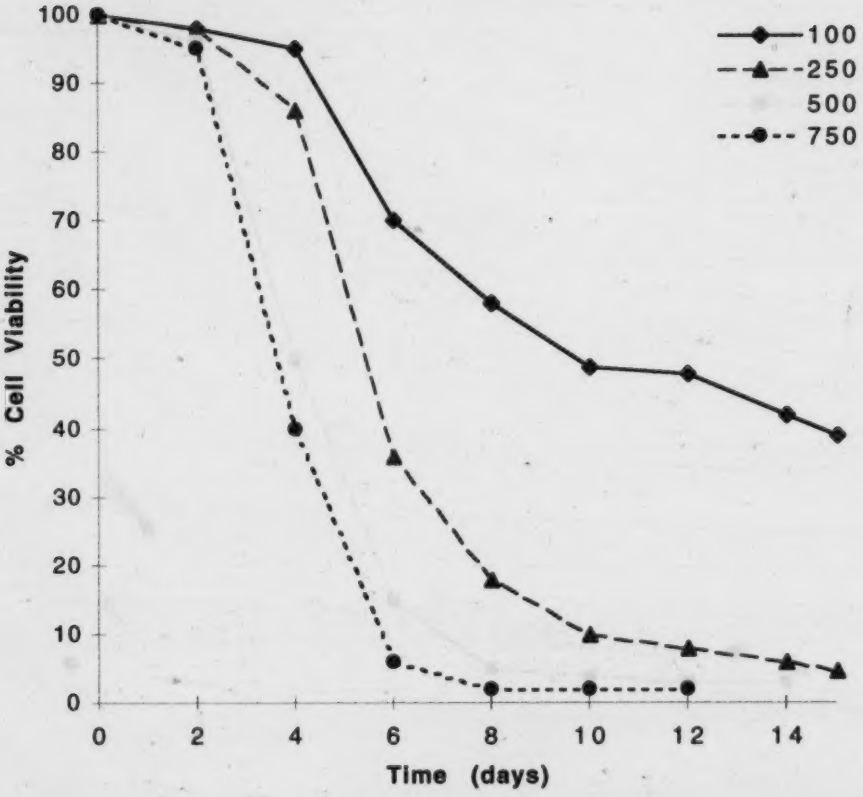


Figure 3.2 Growth of HepG2 cells in varying concentrations of Zeocin™. Cells were incubated in media containing Zeocin™ at the concentrations (μg/ml) shown in the figure. Cell viability was determined using trypan blue exclusion as described in Materials and Methods.

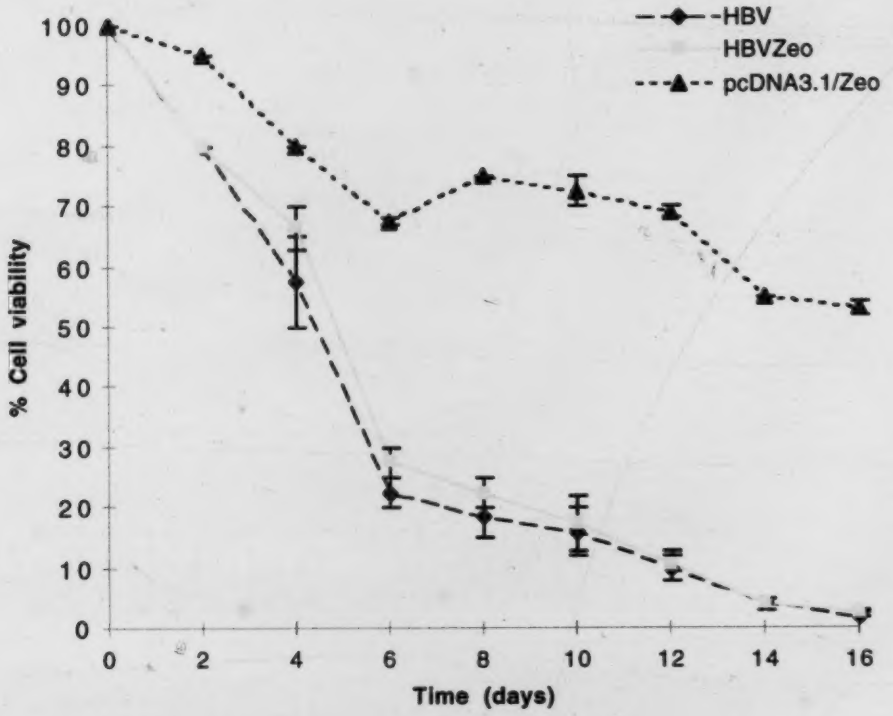


Figure 3.3 Growth of HepG2 cells transfected with HBV, HBVZeo and pCDNA3.1/Zeo in 250 μg/ml Zeocin™ plus media. Transfections were performed as described in Materials and Methods. Transfected cells were incubated in normal media for 48 hr to allow protein expression and then trypsinized and grown on the selective media. Cell viability was determined using trypan blue exclusion and quantified in duplicate using a hemocytometer. The data are presented as the mean of two individual experiments with standard deviations.

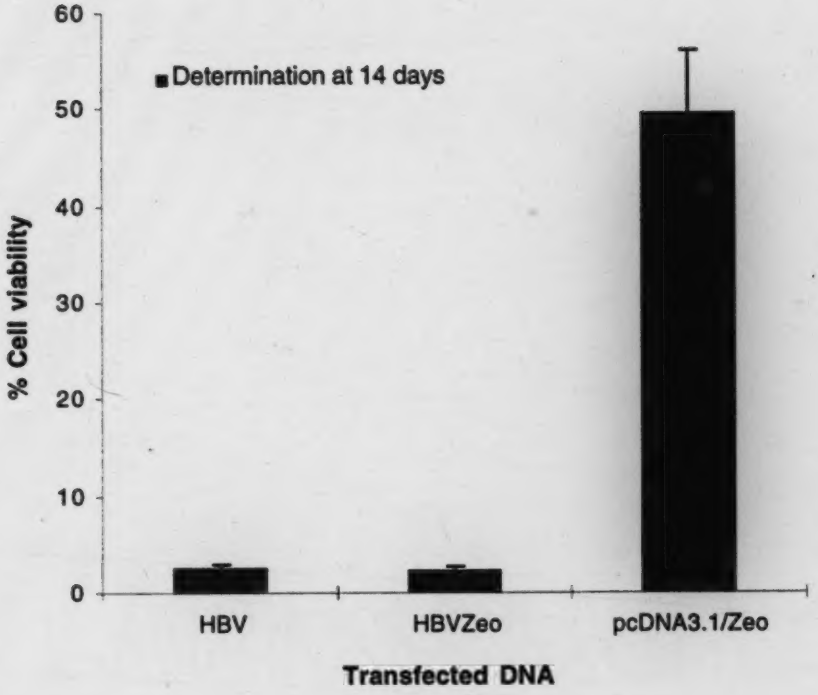


Figure 3.4 Percentages of viable HepG2 cells transfected with HBV, HBVZeo and pCDNA3.1/Zeo and incubated in media containing 250 µg/ml Zeocin™. Fourteen days after growing in the selective media, cells were harvested. The number of viable cells in each sample was determined in duplicate using trypan blue exclusion and a hemocytometer. The results are presented as an average with standard deviations of the results of four individual experiments.

The replication capability of HBVZeo was investigated by evaluation of the endogenous polymerase activity of its extracellular viral particles as described in Materials and Methods. The reaction products were analyzed by agarose gel electrophoresis and autoradiography. Labeled DNA bands, corresponding to relaxed circular and linear double stranded DNA genome and single stranded DNA, were detected, albeit at very low levels, as a result of the DNA polymerase activity of HBVZeo (Figure 3.5). In comparison with the wild type HBV, the HBVZeo polymerase activity quantified by a phosphoimager was attenuated to about 0.3%.

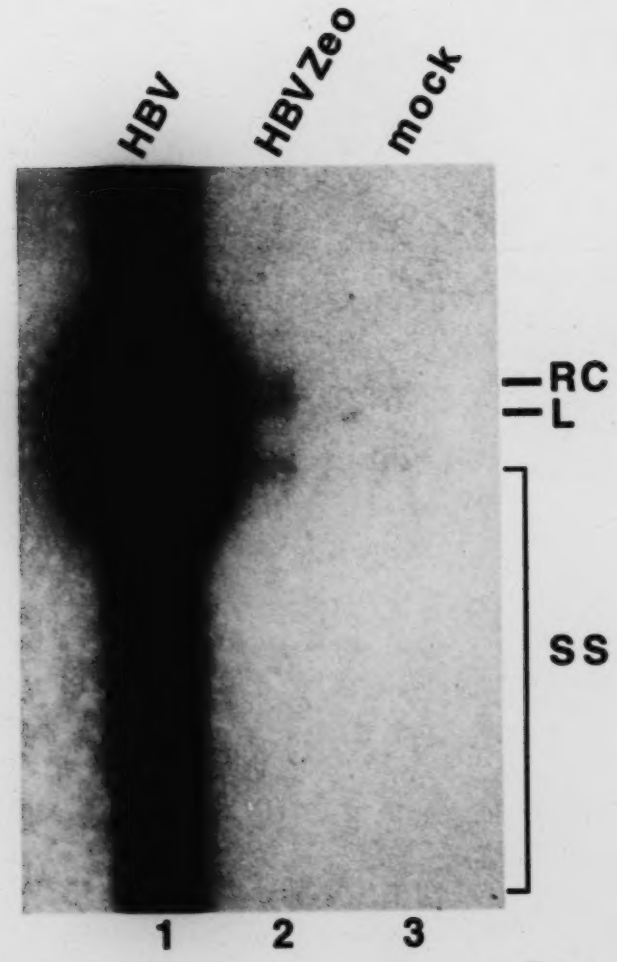
Expression of Zeo^R from the nonreplicative HBVZeoS

To test if a functional Zeo^R protein could be expressed from an HBV nonreplicative vector, HepG2 cells were transfected with HBVZeoS. The percentage of viable cells was determined by the same method as for expression of Zeo^R from HBVZeo. After 14 days of expression in the Zeocin™ containing medium, an average of 10.2% of cells transfected with HBVZeoS survived and was significantly higher than that of cells surviving in samples transfected with HBV, a negative control (Figure 3.6). This significant survival rate of cells transfected with HBVZeoS, about 3.8 times of the negative control, is indicative of the expression of a functional Zeo^R protein from HBVZeoS.

Replication of a nonreplicative HBV vector supported by Pol *trans*-complementation

The ability of a constructed viral vector to produce infectious particles is very important for introducing gene(s) into target cells. Since the expression of Pol was completely ablated in HBVZeoS, an assay to test the possibility of synthesis of complete particles from this nonreplicative HBV construct by *trans*-complementation with Pol was necessary. Endogenous polymerase assays of the extracellular viral particles of HBVZeoS with and

Figure 3.5 Endogenous polymerase activities of replicative HBVZeo. HepG2 cells were transfected with wild type HBV, HBVZeo or a mock plasmid. Extracellular viral particles were harvested and endogenous polymerase activities of the viral particles were determined 4-5 days post-transfection. Extracellular viral particles produced from approximately equal amounts of transfected cells were used in each assay after normalizing to human growth hormone (hGH), an internal control for transfection efficiency. The DNA products were analyzed by agarose gel electrophoresis and autoradiography. RC, relaxed circular; L, linear; SS, single stranded.


HBV
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3

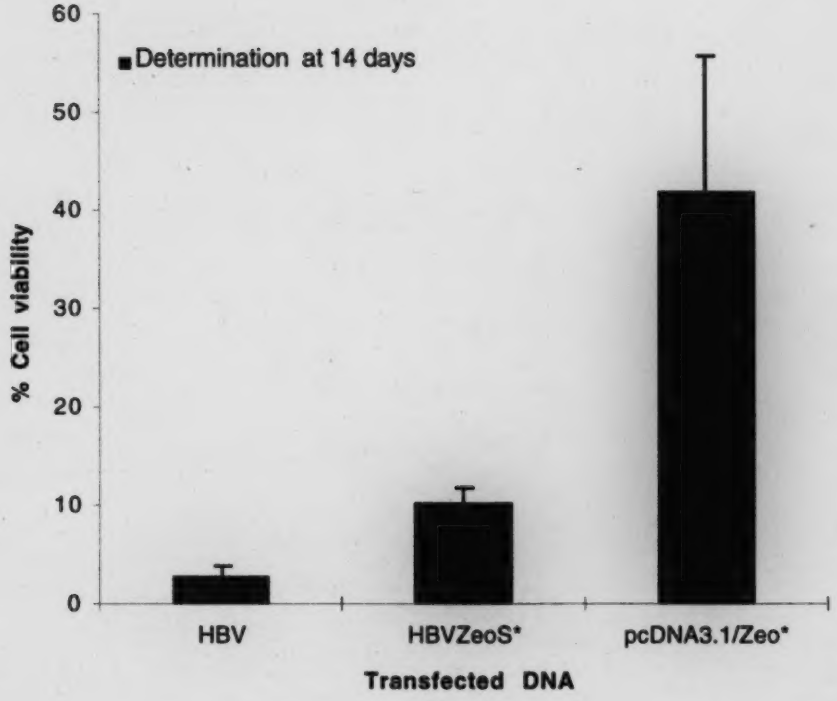

Figure 3.6 Percentages of viable HepG2 cells transfected with HBV, HBVZeoS and pCDNA3.1/Zeo in 250 μg/ml Zeocin™ containing media. Fourteen days after growing in the selective media, the number of viable cells in each sample was determined in duplicate by trypan blue exclusion. The results are presented as an average of four independent experiments with standard deviations. *The increases in the percentages of cell viability were determined to be statistically significant (t-test, $p < 0.05$).

without *trans*-complementation with Pol were carried out. The results showed clearly that the DNA genome was synthesized from HBVZeoS with Pol complementation (Figure 3.7, lane 2 vs. lane 1) and the *trans*-complemented polymerase activity was approximately 1.5-3.0% of that of the wild type HBV.

Expression of HBV genes from HBV replicative and nonreplicative vectors

Expression of HBsAg and HBeAg from HBVZeo and HBVZeoS was quantified by radioimmunoassay. The amounts of HBsAg and HBeAg expressed from HBVZeo were about the same level as that expressed from wild type HBV (Table 3.1). HBsAg expressed from HBVZeoS was also about the same level as that expressed from the wild type but the expression of HBeAg from the HBVZeoS nonreplicative recombinant was reduced to about 60% of that of the wild type (Table 3.2).

Figure 3.7 Endogenous polymerase activities of nonreplicative HBVZeoS *trans*-complemented with Pol. HepG2 cells were cotransfected with wild type HBV, HBVZeoS or a mock plasmid with pCHBVP (a Pol expressing plasmid). Endogenous polymerase activities were determined 4-5 days post-transfection. Extracellular viral particles produced from approximately equal amounts of transfected cells were used in each assay after normalizing to human growth hormone (hGH), an internal control for transfection efficiency. The DNA products were analyzed by agarose gel electrophoresis and autoradiography.

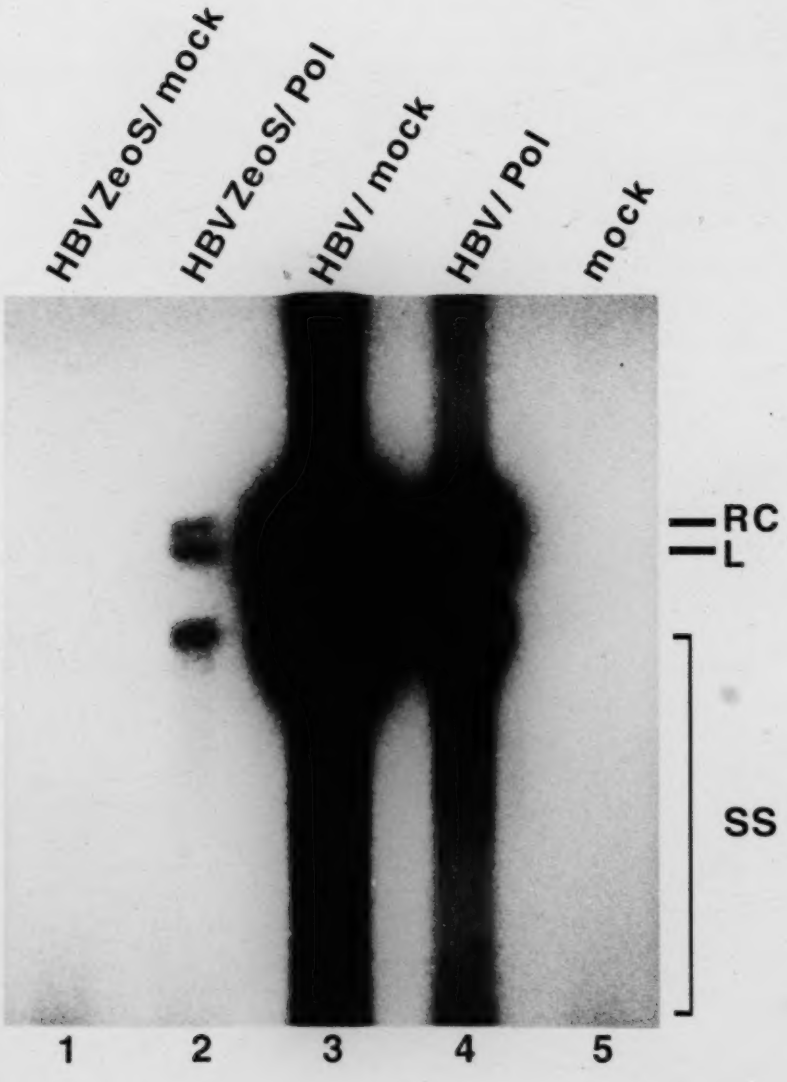
HBVZeoS/ mock
HBVZeoS/ Pol
HBV/ mock
HBV/ Pol
mock
RC
L
SS
1
2
3
4
5

Table 3.1 Detection of HBsAg and HBeAg produced by HBVZeo

Samples[a]	Amounts (S/N[b] ± standard deviation)	
	HBsAg	HBeAg
HBV	115.87 ± 3.29	157.11 ± 40.58
HBVZeo	107.29 ± 6.48	137.83 ± 46.79
mock	1.24 ± 0.10	1.19 ± 0.07

[a] Culture media from HepG2 cells transfected with wild type HBV, HBVZeo or mock transfected were assayed for HBsAg and HBeAg by MEIA.

[b] HBsAg and HBeAg produced were determined as S/N values as described by the manufacturer of the kit used for the assays. ≥ 2.00 S/N is the cutoff rate for positive results. According to the manufacturer, ≥ 7.00 S/N of HBsAg detected is equivalent to 4-15 ng/ml concentration but the absolute concentration of HBeAg is not determined.

Table 3.2 Detection of HBsAg and HBeAg produced by HBVZeoS

Samples[a]	Amounts (S/N[b] ± standard deviation)	
	HBsAg	HBeAg
HBV	138.54 ± 18.47	122.16 ± 26.52
HBVZeoS	131.11 ± 36.94	73.76 ± 12.34
mock	1.32 ± 0.09	1.26 ± 0.14

[a] Culture media from HepG2 cells transfected with wild type HBV, HBVZeoS or mock transfected were assayed for HBsAg and HBeAg by MEIA.

[b] HBsAg and HBeAg produced were determined as S/N values as described by the manufacturer of the kit used for the assays. ≥ 2.00 S/N is the cutoff rate for positive results. According to the manufacturer, ≥ 7.00 S/N of HBsAg detected is equivalent to 4-15 ng/ml concentration but the absolute concentration of HBeAg is not determined.

IV. Discussion

The ideal approach for liver-specific gene transfer *in vivo* requires an efficient gene delivery system for directly targeting genes of desire to the liver. Moreover, the expression of the transgenes should be achieved at therapeutic levels and in an optimal time period. Two important features of HBV favor its use as gene delivery vectors for hepatic gene therapy. Firstly, its replication is highly restricted to liver cells,[23-25] probably via the specific receptor binding, and secondly, the promoter/enhancer elements exhibit a high degree of liver cell specificity.[12-15,23,26,27] Sandig *et al*[15] tried to express the low-density lipoprotein (LDL) receptor by the HBV-CMV hybrid promoter and demonstrated its strong liver specificity even in an adenoviral context. Adenoviral vectors, or retroviral vectors, however, can transduce multiple tissues. An introduction of transgenes to non-targeted tissues may result in undesired consequences, even though the genes may not be expressed. Restriction of both vector targeting and gene expression specifically to the liver using HBV vector systems may therefore be a more favorable approach.

The manipulation of the HBV genome for expression of a foreign gene had not been studied until recently perhaps because of its extremely compact genomic organization and the fact that most of the sequences seem to be indispensable. I have demonstrated that HBV can be manipulated as a gene delivery vector.[16] I have been able to express the HIV-1 *tat* gene in the HBV context in hepatic and nonhepatic cells. The expression of the functional HIV-1 *tat* protein (Tat) seems to be regulated by the preS1 promoter and/or the internal initiation of the pregenomic RNA expressed by the C promoter. The expression is stronger in HepG2 cells than in HeLa or LMH cells. Thus, it is evident that the expression of the pregenomic and preS1 transcripts of HBV are highly liver-specific.[12,13,26-29]

In this study, I have further investigated on the possibility of expressing a gene larger than HIV-1 *tat*, or Zeo^R, by a replicative vector. Based on the indirect analysis for the expressed Zeo^R protein by cell viability assays, I conclude that no functional Zeo^R protein

seems to be produced from the expression of the HBV replicative vector. Two possibilities could be hypothesized on the basis of these results. First, the Zeo^R protein expressed as a fusion form by the HBV replicative vector may not be active since its C-terminus is fused with the Pol protein, which might conceivably affect a structural configuration required for the antibiotic binding.[19] Second, the functional Zeo^R protein might be expressed but not in sufficient quantities to confer resistance to the antibiotic and, therefore, could not be detected by this assay. These possibilities, however, have been addressed by the ability to express the functional Zeo^R protein by a HBV nonreplicative vector under the control of the same regulatory elements as in the HBV replicative construct. Consequently, it is implicated that the C-terminus of the Zeo^R protein is probably essential for the mechanism of action of the protein in binding to the Zeocin™ antibiotic.

The higher number of the survival rate of HepG2 cells transfected with the positive pcDNA3.1/Zeo plasmid than that of cells transfected with a HBVZeoS nonreplicative recombinant may result from the higher protein expression by the CMV regulatory elements from the positive plasmid than tat expressed by the HBV promoter. It is also possible that the high amount of the Zeo^R protein produced from this positive plasmid may cause a bystander effect by sequestering a large quantity of the antibiotic from the media, thus reducing the concentration of the antibiotic to lower than an optimal level. Therefore, the nontransfected cells surrounding the Zeo^R protein producing cells may also be rescued. The HBVZeoS-transfected cells, however, appear to be able to express only sufficient quantities of the resistant protein to exhibit the resistant phenotype.

The insertion of this Zeo^R gene into the HBV replicative vector has reduced its replication capacity dramatically to about 0.3% of the wild type level. This is lower than that found in the HIV-1 *tat* insertion[16] which is about 1.5% of the wild type. This data, however, indicates that complete vector particles can be produced from the HBV replicative construct. Further studies will be necessary to determine whether or not the titer of the recombinant vector particles produced is sufficient to confer efficient gene transfer.

Based on these data, some points on the development of an HBV replicative vector can be made. It appears that the size of a gene to be expressed by this type of vector will be limited because insertion of a large gene seems to have significant effect on replication and/or packaging of the vector. Moreover, the nature of the functional mechanism of the transgenes to be expressed has to be put into consideration because a HBV replicative vector expresses an inserted gene in a fusion form which may have alteration in its therapeutic activity. These limitations may favor the development of HBV as nonreplicative vectors for liver gene transfer.

This study introduces a promising strategy of exploiting HBV nonreplicative vectors as a means to deliver genes to the liver. The feasibility of constructing an HBV nonreplicative vector and of expressing a foreign gene, *i.e.* the Zeo^R, by this type of vector has been demonstrated. Since replication of the nonreplicative HBV recombinant can be supported by *trans*-complementation with Pol, this has offered a potential means of expressing a large foreign gene by the HBV vector. The significant reduction in HBeAg secretion might be advantageous for the use of an HBV nonreplicative vector in a therapeutic application. It has been speculated that HBeAg may be involved in eliciting a host immune-response.[30,31] The reduced HBeAg production by the HBV nonreplicative vector might, therefore, diminish the problem of a host immune-response which may lead to the destruction of the transduced cells. This has been a problem of adenoviral vectors.[32-35]

In summary, an HBV nonreplicative vector may be a better alternative than an HBV replicative vector for therapeutic application due to the safety reason, the nature of a functional protein to be expressed and the payload concern for expression of a large gene. An intensive study into this vector system is warranted.

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CHAPTER 4*

Hepatitis B virus-mediated expression of chicken anemia virus VP3 gene (Apoptin) in human hepatoblastoma cells

I. Introduction

Recent advances in recombinant DNA technology have provided new possibilities for utilization of genetic elements or products as targets for gene therapy of genetic and acquired liver diseases.[1,2] Gene therapy strategies are achieved by exploiting gene delivery vectors. Of a number of vector types which have been developed, viral vectors seem to be the most efficient systems for both *ex vivo* and *in vivo* use.[3] Retroviral and adenoviral vectors have been investigated extensively as gene delivery systems for liver-directed gene therapy. However, several drawbacks to the use of these systems have been described.[1,4,5] For example, recent *in vivo* studies using adenoviral transfer in gene therapy for hepatocellular carcinoma have revealed that this vector exhibits poor transduction ability to hepatoma cells.[6,7] For *in vivo* gene therapy of the liver, therefore, efficient gene delivery systems for specifically directing gene expression to hepatocytes are not yet available.

Because of its strong liver specificity and suitability for both transduction and transcription abilities,[8-10] hepatitis B virus (HBV) appears to be an excellent candidate to mediate gene expression in the liver. The development of HBV vectors for liver gene therapy or for the treatment of liver cancers, is still in its infancy. Most of the studies on HBV in gene therapy have focused on the exploitation of HBV promoter/enhancer elements alone for specifically regulating the expression of transgenes in the liver.[10,11] Our laboratory, however, has manipulated the entire HBV genome as replicative and nonreplicative vectors for both targeting and regulating of specific gene expression for

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hepatic gene therapy.[12] The expression of a transgene, the *tat* gene from human immunodeficiency virus type 1 (HIV-1), in the HBV vector has been demonstrated.

In the present study, these HBV vectors have been further characterized to investigate the possibility of employing these systems in therapeutic application for gene therapy of liver cancers.

Hepatocellular carcinoma is one of the most common tumors in the world for which the conventional therapies are not satisfactory and the prognosis is poor. A common mechanism underlying liver carcinomas as well as other malignancies is the aberrant regulation of cell growth and development which may result from specific mutations that activate cellular oncogenes or inactivate tumor suppresser genes.[13] The identification of genes critical to the initiation and progression of malignancies presents a powerful framework for devising strategies for prevention, diagnosis and treatment, particularly by gene therapy.[14-17] Using this approach, a particular gene(s), which is involved in carcinogenesis, can be efficiently introduced into tumor cells and the normal mechanism of that gene(s) could be restored. However, most malignancies, including liver tumors, arise as the result of multiple events which synergistically alter the regulation mechanisms of cell growth. This raises the question of whether or not correction of only particular gene(s) would be effective for cancer treatment.

Recent data suggest that alterations which cause an imbalance between cell proliferation and apoptosis have a profound impact on the survival and growth of mutated or preneoplastic cells and on their progression into tumors.[18-21] Apoptosis, therefore, plays an important role in the pathogenesis of cancers. Direct induction of cancer cells to undergo apoptosis may be one of the suitable approaches for effective cancer treatment.

Recently, Noteborn and colleagues have discovered that the protein encoded on the VP3 gene of chicken anemia virus (CAV) can induce cellular apoptosis.[22-24] This CAV-VP3 protein or so-called apoptin is a 14 kD proline-rich basic protein which exhibits potent apoptotic activity in chicken and human malignant cells but not in normal cells.[25] Its

activity is independent of p53 expression[24] and cannot be inhibited by the antiapoptotic activity of *bcl*-2.[26] The CAV-VP3 gene, therefore, is a candidate for cancer gene therapy.

In this study, the expression of the potential antitumor gene, the CAV-VP3, by both replicative and nonreplicative HBV vectors was determined in human hepatoblastoma cells. The characteristics and apoptotic inducibility of the protein expressed by both types of vectors were examined. Although an HBV nonreplicative vector may be more favorable for therapeutic use, an attenuated replication-competent HBV vector may also be useful.

II. Materials and Methods

Plasmid construction

A CAV-VP3 expressing plasmid, pHEF-VP3, was constructed by Dr. Y. Cui (Dr. L.-J. Chang's laboratory) by subcloning the CAV-VP3 gene from pCAA-5[27] (kindly provided by Dr. D. Todd; Veterinary Sciences Division, Stormont, Belfast, Northern Ireland) into *Eco*RI/*Bam*HI sites of pHEF1αBSD7 (constructed by Dr. D. Daney; Dr. J. F. Elliott's laboratory). The subcloning was performed by PCR using the upstream primer 5' GGA-ATTCCACCATGAACGCTCTCCAAGAAG 3' and the downstream primer 5' TTGGAT-CCATCTTACAGTCTTATAC 3'. The expression of the CAV-VP3 gene by pHEF-VP3 was controlled by the human elongation factor 1α promoter (HEF 1α).

To construct a replicative HBVVP3 recombinant, the CAV-VP3 gene was subcloned without a stop codon from pHEF-VP3 into the unique *Bst*EII site in-frame with the HBV polymerase gene (P) of pTHBV.[12] The subcloning was performed by PCR as described in Chapter 2 using the upstream primer 5' TGCGGGTCACCAATGAACGCTCTCCAA-GAAGAT 3' and the downstream primer 5' TTCCCAAGAATATGGTGACCCCAGTCT-TATACACCT 3' (the *Bst*EII sites are underlined). DNA sequencing analysis confirmed the actual sequence. A replication-competent form (pTHBVVP3-d) carrying two copies of a head to tail tandem configuration of the HBVVP3 monomer was further constructed and used for the study.

A nonreplicative HBVVP3S recombinant was also constructed by PCR for subcloning the entire CAV-VP3 open reading frame (ORF) with a stop codon into the unique *Bst*EII site in the P gene of pTHBV.[12] The upstream primer 5' TGCGGGTCACCAATGAACG-CTCTCCAAGAAGAT 3' and the downstream primer 5' CCAAGAATATGGTGACCC-TTACAGTCTTATACACCT 3' (the *Bst*EII sites are underlined) were used. DNA sequencing confirmed the actual sequence. Since the insertion contained a stop codon that

was in-frame with the P gene, the polymerase protein (Pol) expression was eliminated. A dimeric form of HBVVP3S (pTHBVVP3S-d) was constructed by ligation head to tail of two copies of the HBVVP3S monomer in the pT7T318U vector (Pharmacia Biotech, Uppsala, Sweden) and used for the study.

pHEF-GFP, pHEF-IFN-γ and pHEF-vpr (kindly provided by Dr. L.-J. Chang) were expressing plasmids used for expression of green fluorescence protein (GFP), gamma-interferon (IFN-γ) and HIV-1 vpr, respectively. pTHBV-d and pTHBVZeo-d were constructed as described in Chapters 2 and 3, respectively.

Cell culture

Human hepatoblastoma cells (HepG2) were cultured and maintained at 37°C in 5% CO_2 in Auto-Pow MEM Eagle (modified) medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 10 mM sodium bicarbonate, 2 mM L-glutamate, 10% fetal bovine serum, 50 units/ml penicillin G sodium, 0.01 mg/ml streptomycin and 50 units/ml nystatin. Human foreskin fibroblast cells (HFS) (Advanced Biotechnologies Inc., Columbia, MD, USA) were also maintained at 37°C in 5% CO_2 in the same culture medium as HepG2 cells.

Transfection

Transfections were performed using Lipofectin (GIBCO BRL Life Technologies, Gaithersburg, MD, USA) and microparticle bombardment.

Transfection using Lipofectin

Transfections were performed as recommended by the manufacturer with some modifications. A total amount of 3.5 μg of DNA per a 35-mm tissue culture dish and a 1:6 ratio of DNA and Lipofectin were used. Cells were subcultured 20 hr prior to transfection

and were about 60-70% confluent at the time of transfection. Cells were fed with fresh media 1 hr before transfection. The plasmid DNA and Lipofectin were each diluted with unsupplemented medium (no FBS nor antibacterial agents). These two solutions were combined, incubated for 15-30 min at room temperature, and then applied to cells which had been washed twice with the unsupplemented medium. The cells were incubated at 37°C in 5% CO_2 for 4 hr. An equal volume of the normal medium without supplementation of antibacterial agents was then added with further incubation for about 20 hr. Cells were washed twice and fed with normal media.

Transfection by microparticle bombardment or Gene Gun technique

The procedure was modified and performed by Dr. A. Gainer (Dr. J. F. Elliott's laboratory).

Precipitation of DNA onto gold particles: To 25 mg of 1.6 μm gold particles (Bio-Rad Laboratories, Mississauga, ON, Canada), 100 μl of 0.05 M spermidine, 50 μg of DNA (optimal concentration ~1 μg/μl) and 100 μl of 1 M $CaCl_2$ were added. The solution was mixed and incubated at room temperature for 10 min to allow precipitation. The pellet was isolated by microcentrifugation and washed three times with absolute ethanol (100%). The pellet was then resuspended in 200 μl of 0.1 mg/ml polyvinyl pyrollidone (PVP) in absolute ethanol and then diluted to the final volume of 3.0 ml with the PVP solution.

Preparation of DNA-gold-coated tubing: The Tubing Preparation Station from Bio-Rad was used. The tubing (Bio-Rad) was purged for at least 15 min with nitrogen gas. The DNA-gold suspension was aspirated into the tubing using a syringe. The tubing was then slid into the sleeve of the Tubing Preparation Station and the coating particles were allowed to settle for 3-5 min. Ethanol was aspirated out of the tubing, which was rotated for about 30 sec and then dried with nitrogen gas for 3-5 min. The tubing on which DNA-gold particles were distributed evenly was cut into pieces of 0.5 inch in length and stored at 4°C in a desiccated container ready for use.

Transfection of cells using Gene Gun: Cells were grown to 95-100% confluent in 35-mm tissue culture dishes (Falcon 3001; Becton Dickinson and Company, Lincoln Park, NJ, USA). The helium pressure of the Gene Gun machine was set at 200 psi and a DNA-gold-coated piece of tubing was loaded into the gun. Media were removed from the cells and a cell strainer (40 μm mesh, Falcon) was subsequently placed in an inverted position on top of the cells to dissipate the force of the helium blast. The culture dish was placed on the platform of the Gene Gun. The spacer and body of the Gene Gun containing the DNA-gold-coated piece of tubing were then assembled on top of the culture dish. Transfection was performed by pushing the switch on the electronic box to release a 40 msec blast of helium which introduced DNA-gold particles into the cells. The cell strainer was removed and the cells were fed with fresh media.

To assess transfection efficiency, all transfections were performed in the presence of human growth hormone plasmid pXGH5 (Nichols Institute Diagnostics, San Juan, Capistrano, CA, USA). Secreted human growth hormone (hGH) was quantitated by radioimmunoassay using a HGH-TGES transient gene expression kit (Nichols Institute Diagnostics) at 48 hr after transfection.

For the study of expression of CAV-VP3 using indirect immunofluorescence assay as a detection method, transfections were performed on cells grown on poly-L-lysine treated coverslips.

For transfections using green fluorescence protein (GFP) as a reporter, the GFP expressing plasmid (pHEF-GFP) was cotransfected with a plasmid of interest at a ratio of 2:5 per 35-mm tissue culture dish.

The expression of HBeAg and HBsAg from the HBV plasmids were determined by a Microparticle Enzyme Immunoassay (MEIA) (Abbott Laboratories, Abbott Park, IL, USA).

Nuclear staining for cellular apoptosis using Hoechst 33258

Cells were harvested by trypsinization, transferred to a 15-ml conical tube and centrifuged gently in a Sorvall RT6000B Refrigerated Centrifuge (Dupont, Mississauga, ON, Canada) at 300 g at 4°C for 10 min. Cells were washed once with phosphate-buffered saline solution (PBS). The washing was removed by gentle centrifugation. Cells were resuspended in 4.0% paraformaldehyde in PBS, incubated at room temperature for 10 min and washed once with PBS. Cells were then transferred to a brown-Eppendorf tube using PBS and centrifuged at 300 g at 4°C for 10 min to remove the last drop of the washing. The pellets were resuspended in 10-20 µl of PBS containing 32 µg/ml Hoechst 33258 (bis-benzimide; Sigma Chemical Co.) depending on a number of cells and incubated at room temperature for 15 min. Cells were kept at 4°C until they were analyzed for nuclear apoptosis under a fluorescence microscope (Axioskop Zeiss, Germany) (Carl Zeiss Canada Ltd., North York, ON, Canada). Nuclear staining with Hoechst 33258 (excitation/emission maxima = 352/461 nm) was visualized using a blue DAPI fluorescence filter. Cellular apoptosis was identified through typical apoptotic nuclear fragmentation. Quantitative analyses were performed by scoring an apoptotic rate of at least 500 cells per each quantitation.

Apoptosis induction using cycloheximide

Cells were grown for about 48 hr until they reached 80% confluent. Normal media were then replaced with media containing 100 or 200 µg/ml cycloheximide (Sigma Chemical Co., St. Louis, MO, USA). For long incubation periods, the cycloheximide containing media were changed every 2 days. The media removed from each individual sample were kept at 4°C and the floating cells were ultimately harvested and pooled with adherent cells harvested from the same sample for further detection of cellular apoptosis by nuclear staining using Hoechst 33258.

Quantitation of cellular apoptosis induced by CAV-VP3 expression using GFP as a reporter

Cells were cotransfected with CAV-VP3 expressing plasmids and pHEF-GFP. At various time periods post-transfection, cells were harvested and stained with Hoechst 33258. Induction of apoptosis in transfected cells was evaluated under a fluorescence microscope (Axioskop Zeiss, Germany) using a combination of a green FITC fluorescence filter for the GFP expression (excitation/emission maxima = 395/509 nm) in transfected cells and a blue DAPI fluorescence filter for detecting nuclear fragmentation by the Hoechst staining. Quantitative analyses were performed by counting blindly at least 200 transfected cells per examination. The percentages of transfected cells undergoing apoptosis or the apoptotic rates of transfected cells were determined as:

$$\frac{\text{Number of the transfected cells undergoing apoptosis}}{\text{Number of transfected cells counted}} \times 100\%$$

Indirect immunofluorescence for CAV-VP3 expression

Transfected cells grown on coverslips were washed twice with PBS. Cells were fixed by incubating with 4.0% paraformaldehyde at room temperature for 10 min and washed three times with IF buffer (2.0% fetal calf serum and 0.02% sodium azide in PBS). After washings, cells were incubated with acetone:methanol (1:1) solution at -20°C for 5 min and washed three times with IF buffer. Mouse ascites anti CAV-VP3 monoclonal antibody (kindly provided by Dr. D. Todd; Veterinary Sciences Division, Stormont, Belfast, Northern Ireland) diluted in IF buffer (1:10000) was added to the washed cells with further incubation at 37°C for 1 hr. The antibody solution was removed and cells were washed three times with IF buffer with shaking for 5 min per each wash. Cells were further incubated with a normal goat serum blocking solution (1:20 ratio in IF buffer) at 37°C for 30 min. After removal of the blocking solution, cells were incubated with 1:200 fluorescein-conjugated goat anti-mouse IgG (Cappel: Organon Teknika Corp., West Chester, PA, USA) in IF buffer at 37°C for 1 hr. Following this step, samples were

covered with aluminum foil to protect them from light. Cells were washed three times with IF buffer with shaking for 5 min per each wash and once briefly with PBS.

To detect cellular apoptosis induced by CAV-VP3 expression, cells were further incubated with PBS containing 32 μg/ml Hoechst 33258 at room temperature for 15 min. The solution was removed. Cells were covered with SlowFade® Light Antifade solution (Molecular Probes, Eugene, OR, USA), sealed with nail polish and viewed under a fluorescence microscope (Axioskop Zeiss, Germany) using a combination of a green FITC fluorescence filter for CAV-VP3 expression and a blue DAPI fluorescence filter for detecting cellular apoptosis. It should be noted that the SlowFade® Light Antifade solution enhances the fluorescence of the Hoechst dye intensively and may cause difficulty in visualizing Hoechst-stained nuclei under a fluorescence microscope.

III. Results

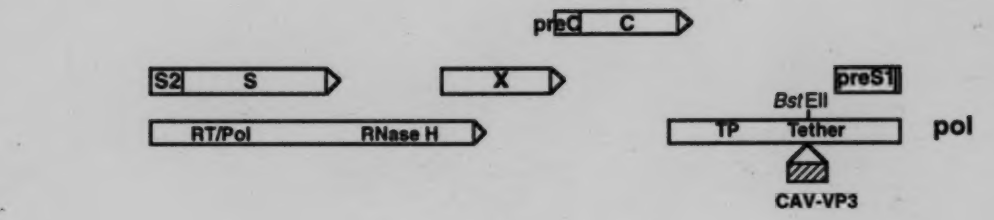
Strategy

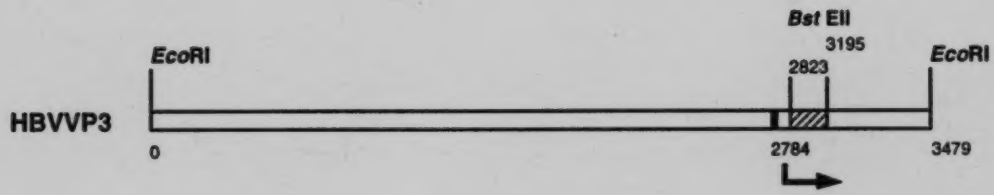
To express the CAV-VP3 in an HBV replicative vector, the gene was inserted without a stop codon in-frame with the P gene. This construct was designated as HBVVP3 (Figure 4.1). The CAV-VP3 gene would be expressed as a fusion form with Pol at the C-terminus. An HBVVP3S replication-incompetent recombinant carried the entire CAV-VP3 gene with its own initiation and stop codon such that the CAV-VP3 gene was to be expressed as a single protein (Figure 4.1). A dimeric construct containing a head to tail tandem configuration of two copies of the HBVVP3 or HBVVP3S monomer was used to study expression of the CAV-VP3 gene from a replicative or nonreplicative HBV vector, respectively. The expression of functional CAV-VP3 protein was determined through induction of cellular apoptosis in transfected human hepatoma cells. HepG2 cells derived from human hepatoblastoma were used for the study.

HepG2 Apoptosis induced by cycloheximide

Prior to the study for the apoptotic ability of the CAV-VP3, apoptosis of HepG2 cells was characterized by treatment of exponentially growing cells with 100 µg/ml or 200 µg/ml cycloheximide for different periods of time. Cycloheximide has been shown to induce apoptosis in some human transformed cells.[28,29] The characteristics of HepG2 cells treated with cycloheximide were examined by staining with Hoechst 33258, a bis-benzimide dye which binds to the chromosomal DNA and emits bright blue fluorescence upon binding.[30] Thus, the cellular nuclei can be visualized under a fluorescence microscope. The hallmarks of cellular apoptosis including nuclear condensation and fragmentation were identified in the cycloheximide treated cells. These typical morphological changes were distinguished from the morphology of normal cells (Figure 4.2). The apoptotic rates of HepG2 after the

Figure 4.1 Schematic representations of HBVVP3 and HBVVP3S. The CAV-VP3 gene was inserted into the unique *Bst*EII site in-frame with the P gene and between the promoter (at the 2784 nucleotide) and the initiation codon of the preS1 gene. An HBVVP3 replication-competent carried the CAV-VP3 gene without a stop codon. An HBVVP3S replication-incompetent contained the entire CAV-VP3 ORF. All the ORFs encoded on the *Eco*RI-*Eco*RI monomer of the HBV genome (3221 bp) with the positions of all initiation codons according to the adw2 subtype are shown. The ORFs start from the blunt end and stop at the arrow end. The four domains of the P (pol) gene, corresponding to the functional activities of the Pol protein, are indicated. The hatched box represents the CAV-VP3 insert. The asterisk indicates a stop codon of the CAV-VP3 gene in HBVVP3S. The solid bar and the arrow indicate the promoter and transcription initiation site of the preS1 gene, respectively. RT/Pol, reverse transcriptase and DNA polymerase; TP, terminal protein.


preC
C
S2
S
X
preS1
BstEII
RT/Pol
RNase H
TP
Tether
pol
CAV-VP3


Bst EII
3195
EcoRI
2823
EcoRI
HBVVP3
0
2784
3479

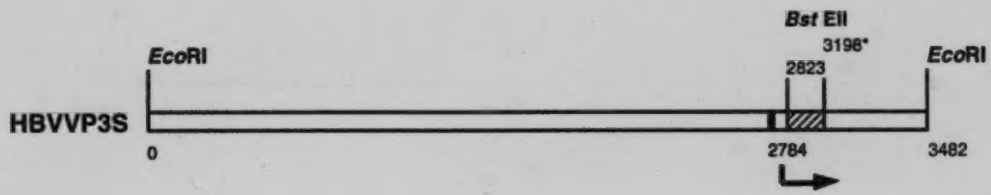
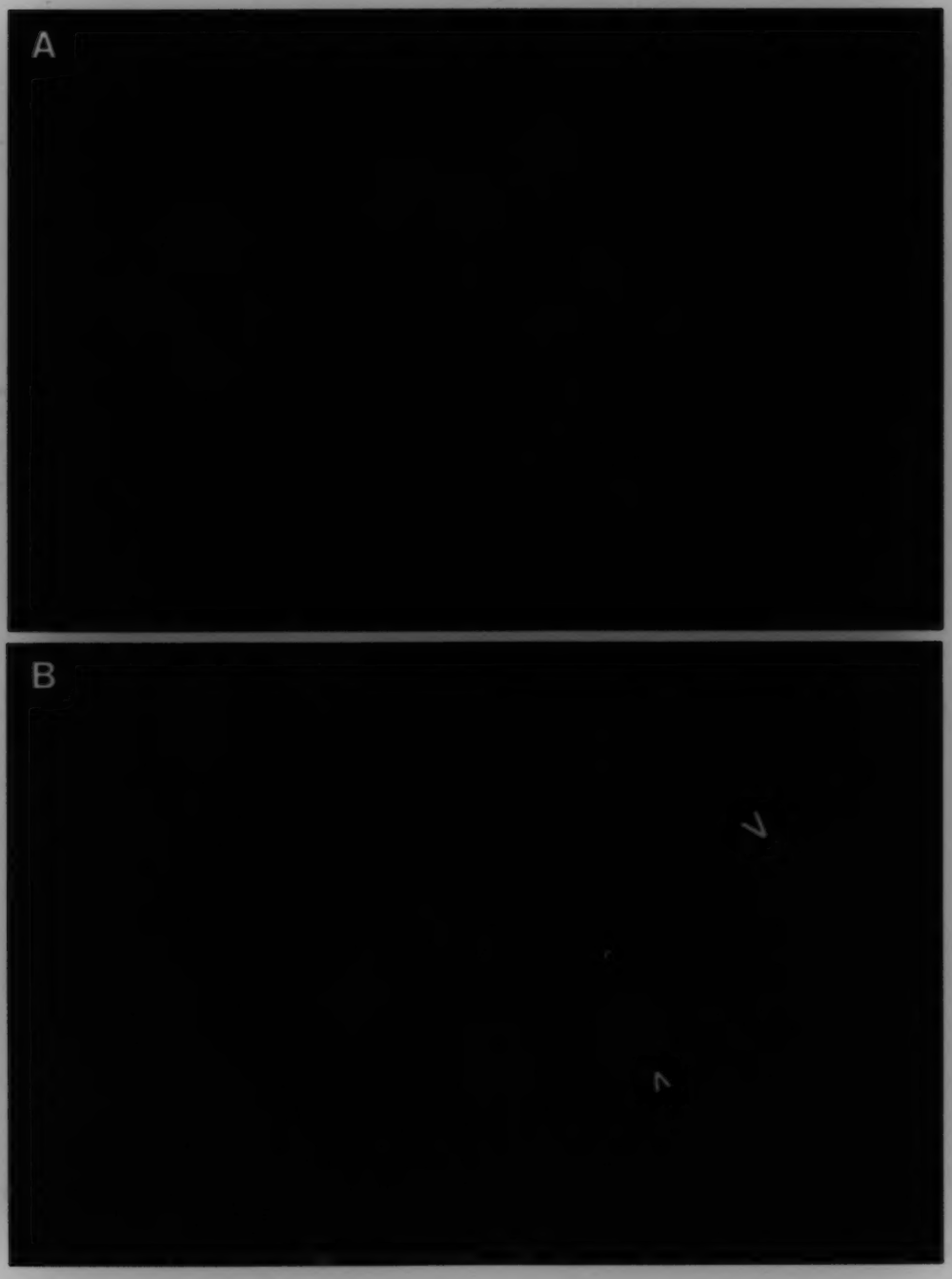

Bst EII
3196*
EcoRI
2823
EcoRI
HBVVP3S
0
2784
3482

Figure 4.2 Apoptotic characteristics of HepG2 cells treated with cycloheximide (CHX). HepG2 cells were treated with 200 μg/ml CHX for 4 days. Cells were then harvested, fixed and stained by Hoechst 33258 as described in Materials and Methods. Nuclear morphology of normal cells (**A**) and apoptotic cells induced by CHX (**B**) were visualized under a fluorescence microscope using a blue DAPI filter. HepG2 apoptosis was identified as nuclear condensation and fragmentation. Small and large arrow heads indicate examples of nuclear condensation and fragmentation, respectively. Original magnification, 1000 X.


A
B

treatments were determined and shown in Figure 4.3. The increase in the concentration or incubation period with the cytotoxic substance enhanced the number of HepG2 apoptosis. Figure 4.4 shows examples of HepG2 apoptosis after treatment with cycloheximide for different periods of time. These data suggested that human hepatoma cells could be triggered to undergo apoptosis.

CAV-VP3 Induces apoptosis in HepG2

To determine whether CAV-VP3 could be expressed and induce apoptosis in human hepatoma cells, the CAV-VP3 expressing plasmid under control of the HEF 1α promoter (pHEF-VP3) was cotransfected with pHEF-GFP into HepG2 cells. Transfections were performed using Lipofectin (two individual sets of experiments) and the Gene Gun technique (one set of experiments). Cotransfected cells, theoretically also carrying the CAV-VP3 plasmid, were identified by detection of GFP expression under a fluorescence microscope. Apoptosis induced in cotransfected cells was simultaneously detected by the typical morphological changes of the cellular nuclei stained by Hoechst 33258.

Based on the assays for the internal control hGH, the transfection efficiency obtained from the two different techniques was not significantly different. The apoptotic rates of the same sample transfected by these different methods were also not different. However, I observed that cells transfected by Gene Gun showed stronger signal of GFP than those transfected by Lipofectin.

The results showed clearly that cells cotransfected with the CAV-VP3 expressing plasmid underwent apoptosis as detected by DNA fragmentation of the cells at 4, 6 and 12 days after transfection (Figures 4.5, 4.6 and 4.7). The apoptotic rate of pHEF-VP3 cotransfected cells was significantly high compared to cells cotransfected with pTHBVZeo-d or a mock plasmid and increased progressively when incubated for longer periods of time

Figure 4.3 Apoptotic rates of HepG2 induced by cycloheximide. HepG2 cells were treated with 100 μg/ml and 200 μg/ml CHX for 2, 4 and 6 days. Cellular apoptosis was identified by nuclear fragmentation and condensation by Hoechst 33258 staining under a fluorescence microscope as described in Materials and Methods. Percentages of HepG2 apoptotic cells in CHX-treated and untreated samples were scored blindly as described in Materials and Methods. Values presented are averages of three independent experiments with standard deviations.

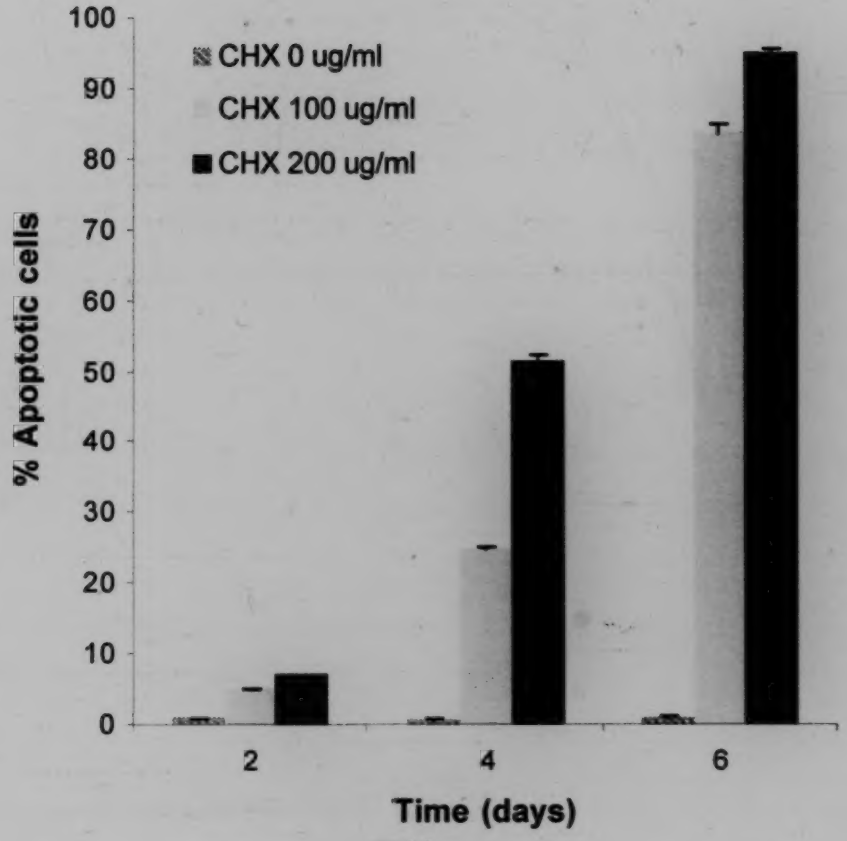

100
90
80
70
60
50
40
30
20
10
0
% Apoptotic cells
CHX 0 ug/ml
CHX 100 ug/ml
CHX 200 ug/ml
2
4
6
Time (days)

Figure 4.4 HepG2 apoptosis induced by CHX treatment. CHX-treated and untreated HepG2 cells were analyzed for cellular apoptosis by Hoechst 33258 staining at 2 days (**A-C**), 4 days (**D-F**) or 6 days (**G-I**) after the incubations. The cellular apoptosis was identified as DNA fragmentation and condensation (arrow heads). **A**, **D** and **G**, without CHX treatment; **B**, **E** and **H**, 100 μg/ml CHX treatment; **C**, **F** and **I**, 200 μg/ml CHX treatment. Original magnification, 200 X.

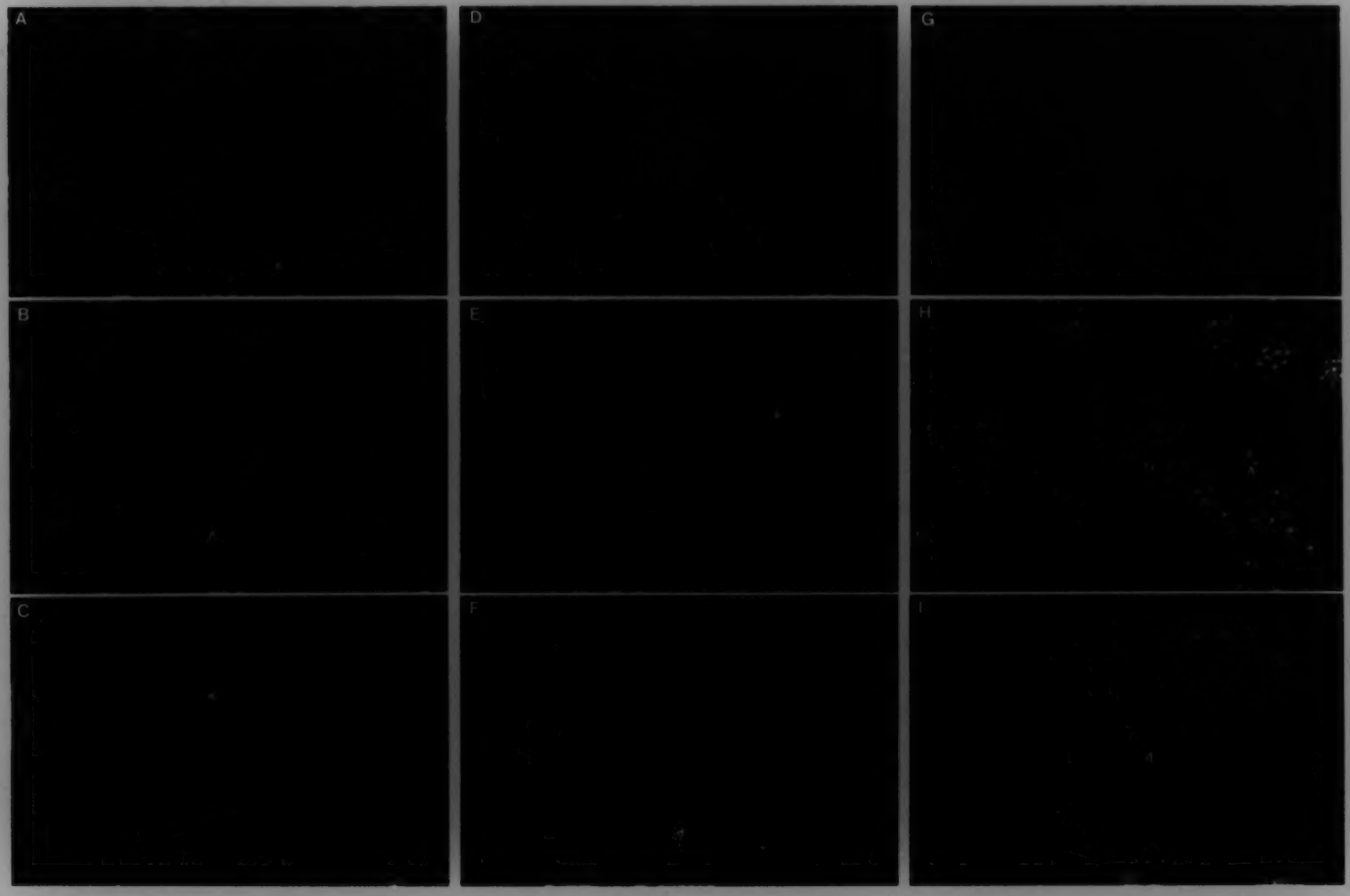

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Figure 4.5 HepG2 apoptosis induced by CAV-VP3 at 4 days post-transfection. HepG2 cells were cotransfected with pHEF-VP3 (**A-C**), pTHBVZeo-d (**D-F**) or a mock plasmid (**G-I**) with pHEF-GFP as described in Materials and Methods. Four days after transfection, cells were harvested and analyzed for apoptosis induction in transfected cells using a fluorescence microscopic method. Transfected cells were detected by GFP expression using a green FITC filter (**A**, **D** and **G**). The nuclei of these cells were identified concomitantly by Hoechst 33258 staining using a blue DAPI filter (**B**, **E** and **H**). Apoptosis induced in the pHEF-VP3 transfected cells was identified as fragmentation of the cellular nuclei. Double exposure of the cells with GFP expression and Hoechst staining are also shown (**C**, **F** and **I**). Original magnification, 1000 X.

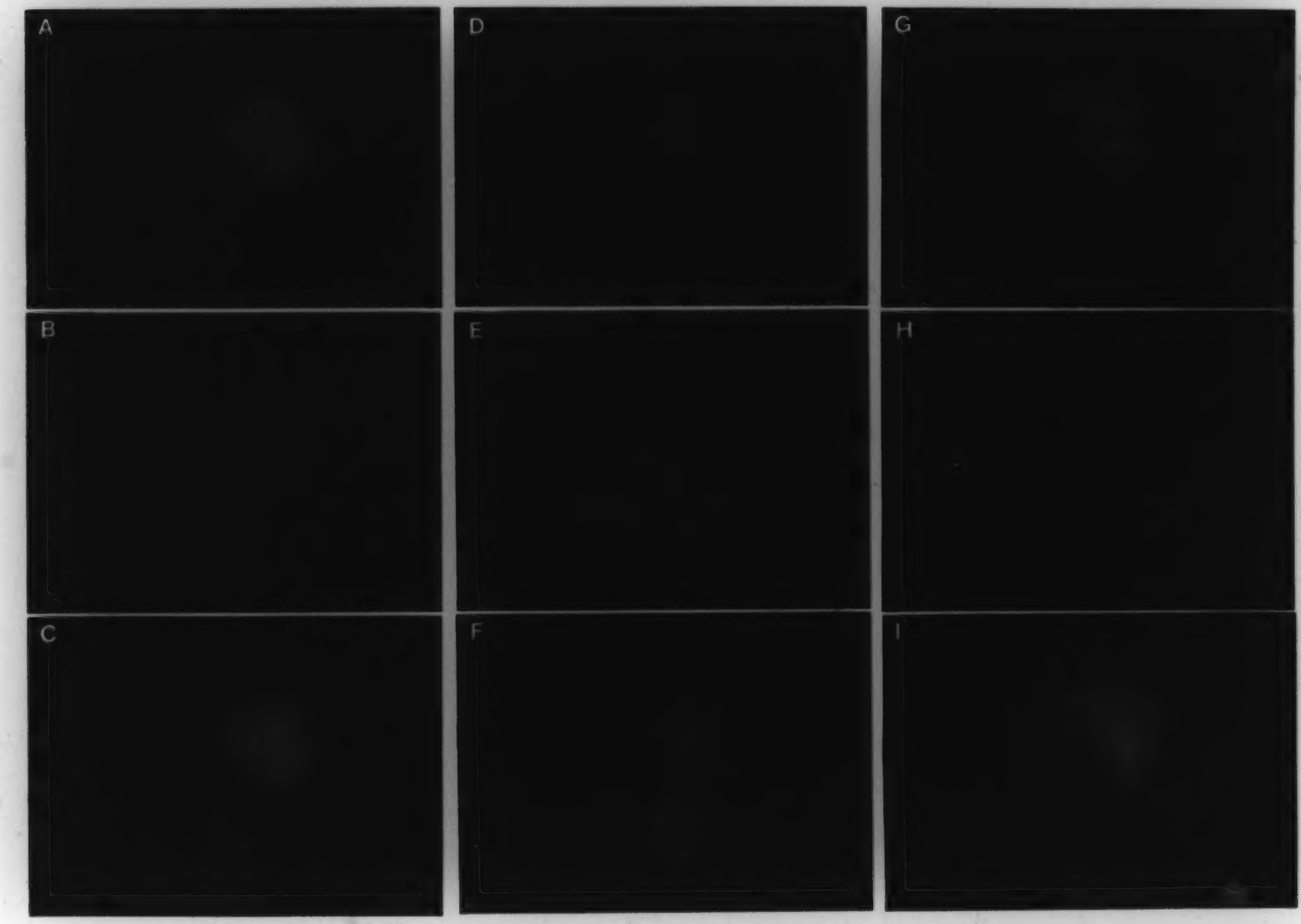
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Figure 4.6 HepG2 apoptosis induced by CAV-VP3 at 6 days post-transfection. HepG2 cells were cotransfected with pHEF-VP3 (**A-C**), pTHBVZeo-d (**D-F**) or a mock plasmid (**G-I**) with pHEF-GFP. Six days after transfection, cells were harvested and analyzed for apoptosis induction in transfected cells using a fluorescence microscopic assay. Transfected cells were detected by GFP expression using a green FITC filter (**A**, **D** and **G**). The nuclei of these cells were identified concomitantly by Hoechst 33258 staining using a blue DAPI filter (**B**, **E** and **H**). Apoptosis induced in the pHEF-VP3 transfected cells was identified as fragmentation of the cellular nuclei. Double exposure of the cells with GFP expression and Hoechst staining are also shown (**C**, **F** and **I**). Original magnification, 1000 X.

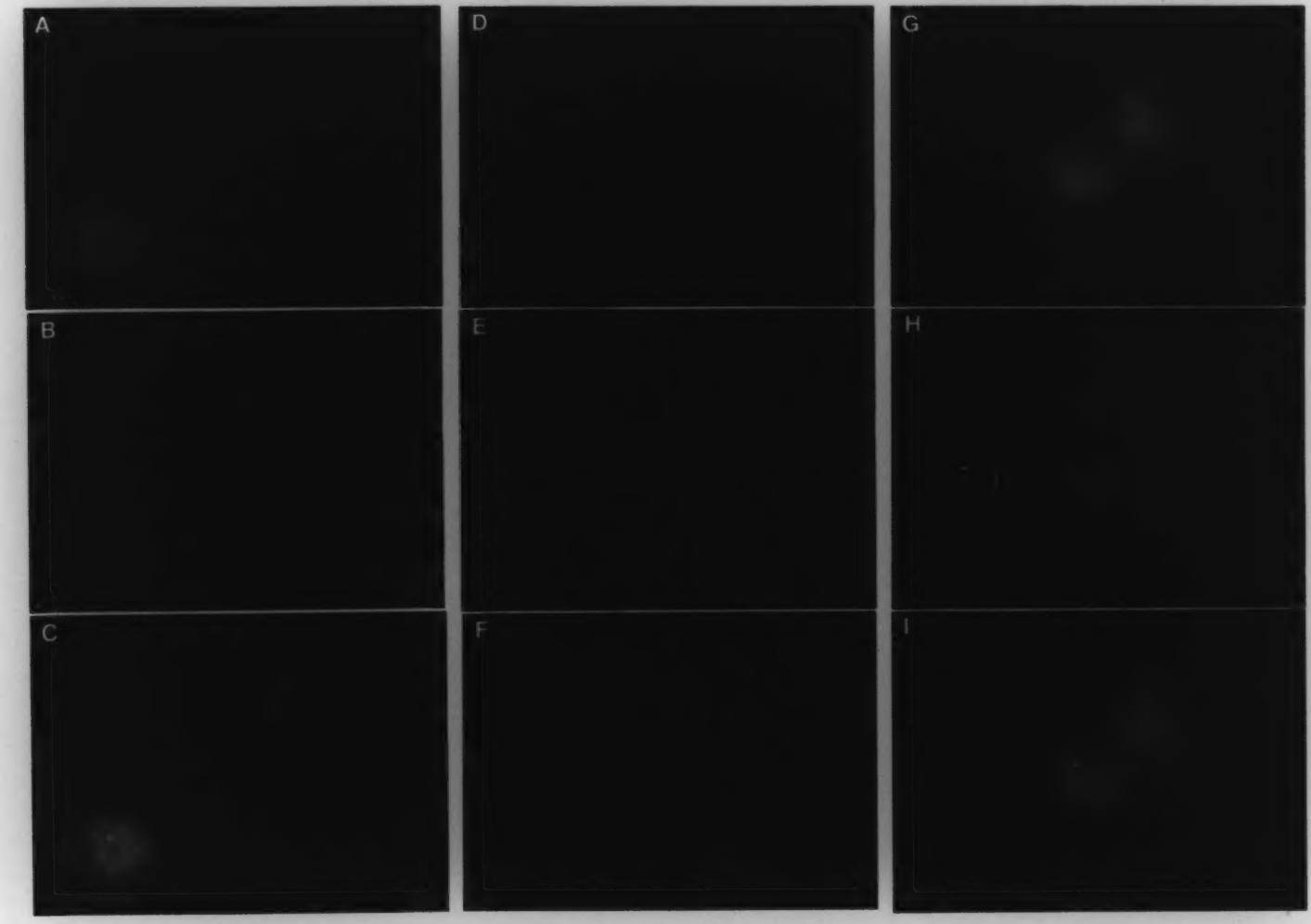
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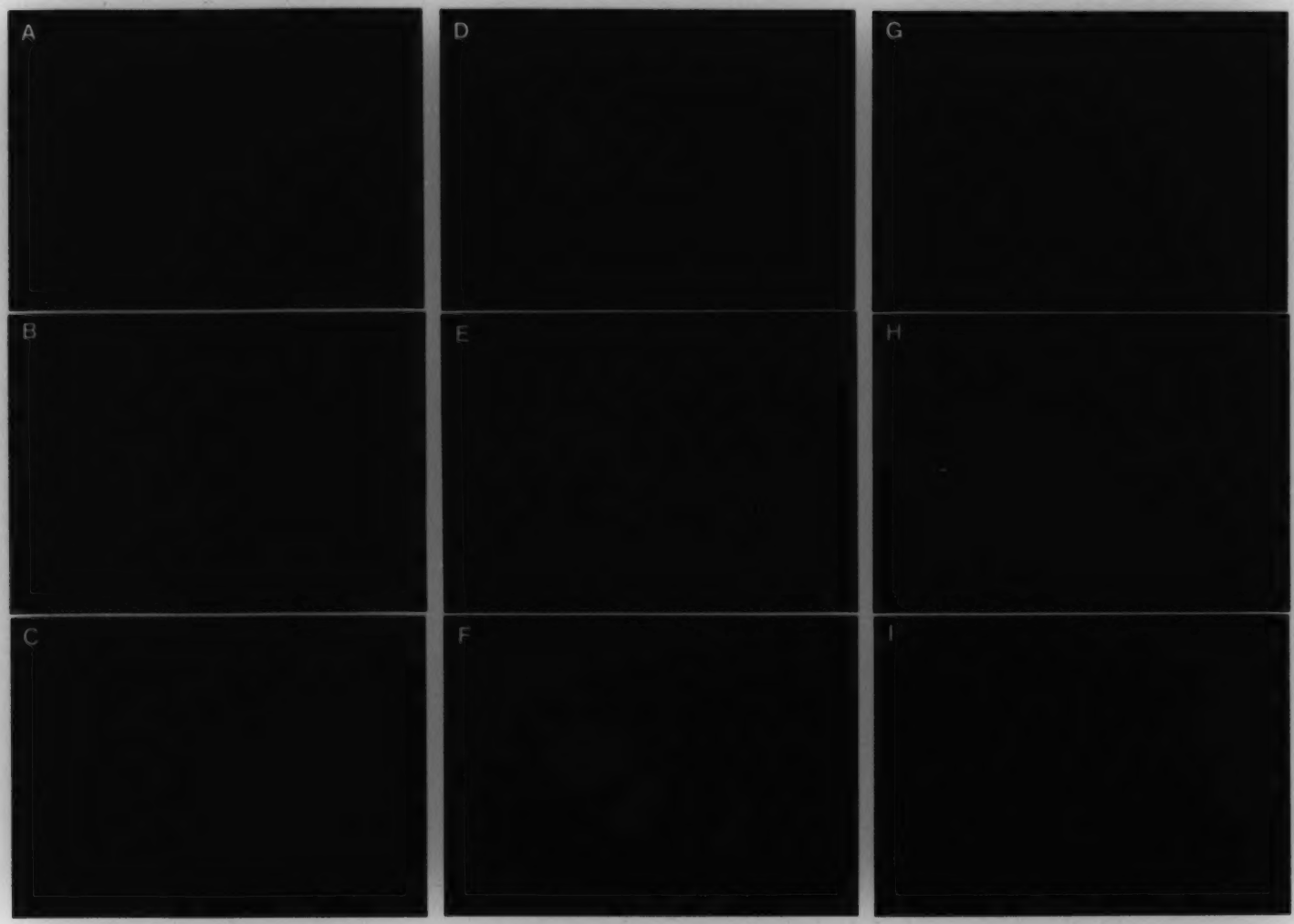
Figure 4.7 HepG2 apoptosis induced by CAV-VP3 at 12 days post-transfection. HepG2 cells were cotransfected with pHEF-VP3 (**A-C**), pTHBVZeo-d (**D-F**) or a mock plasmid (**G-I**) with pHEF-GFP. Twelve days after transfection, cells were harvested and analyzed for apoptosis induction in transfected cells using a fluorescence microscopic assay. Transfected cells were detected by GFP expression using a green FITC filter (**A**, **D** and **G**). The nuclei of these cells were identified concomitantly by Hoechst 33258 staining using a blue DAPI filter (**B**, **E** and **H**). Apoptosis induced in the pHEF-VP3 transfected cells was identified as fragmentation of the cellular nuclei. Double exposure of the cells with GFP expression and Hoechst staining are also shown (**C**, **F** and **I**). Original magnification, 1000 X.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(Figure 4.8). This study indicated that CAV-VP3 induced apoptosis in HepG2 cells. The apoptotic activity of this gene in human hepatoma Hep3B has also been reported.[24]

Cotransfection with pTHBVZeo-d was included to test that an HBV vector carrying a foreign gene about the same length as the CAV-VP3 did not produce significant apoptosis induction in HepG2 cells. The number of apoptotic cells detected in the samples cotransfected with this HBV plasmid was as low as that detected in the mock transfections. Therefore, pTHBVZeo-d could be used as a negative control in experiments testing for expression of the CAV-VP3 by HBV vectors.

Attempts to examine the apoptotic activity of CAV-VP3 in normal human cells

To be used as an antitumor agent, a gene must not be expressed or have any effect in normal cells. Therefore, the apoptotic activity of the CAV-VP3 gene in human foreskin fibroblasts (HFS), nontransformed, nontumor cells, was determined.

The question was first addressed by examining whether cellular apoptosis could be induced in HFS cells. HFS cells were treated with 100 μg/ml or 200 μg/ml cycloheximide for 2,4 and 6 days and induction of apoptosis was determined by Hoechst 33258 staining. The results showed that these nontransformed cells could be induced to undergo apoptosis by cycloheximide. The morphological changes in the HFS apoptosis were observed as the nuclear DNA condensation and fragmentation (Figure 4.9). The number of apoptotic cells increased when the cycloheximide concentration and incubation period were increased (Figure 4.10).

The expression and apoptosis inducibility of the CAV-VP3 in HFS cells was then verified by cotransfecting the CAV-VP3 expressing plasmid (pHEF-VP3) with the GFP plasmid. For negative controls, the cells were cotransfected with pTHBVZeo-d and a mock plasmid. I made many attempts to transfect HFS cells using various procedures, including Lipofectin and Lipofectamine transfection (GIBCO BRL Life Technologies),

Figure 4.8 Apoptotic rates of HepG2 cells induced by the CAV-VP3. HepG2 cells were cotransfected with pHEF-VP3 (HEF-VP3), pTHBVZeo-d (HBVZeo) or a mock plasmid (mock) with pHEF-GFP as described in Materials and Methods. GFP expression and apoptotic induction in transfected cells were examined as described in the legend to Figure 4.5. Percentages of apoptosis induced in transfected cells at 4, 6 and 12, days post-transfection were determined as described in Materials and Methods and presented as averages of three individual experiments with standard deviations. *The percentages of apoptosis were statistically significant (ANOVA, $p < 0.01$).

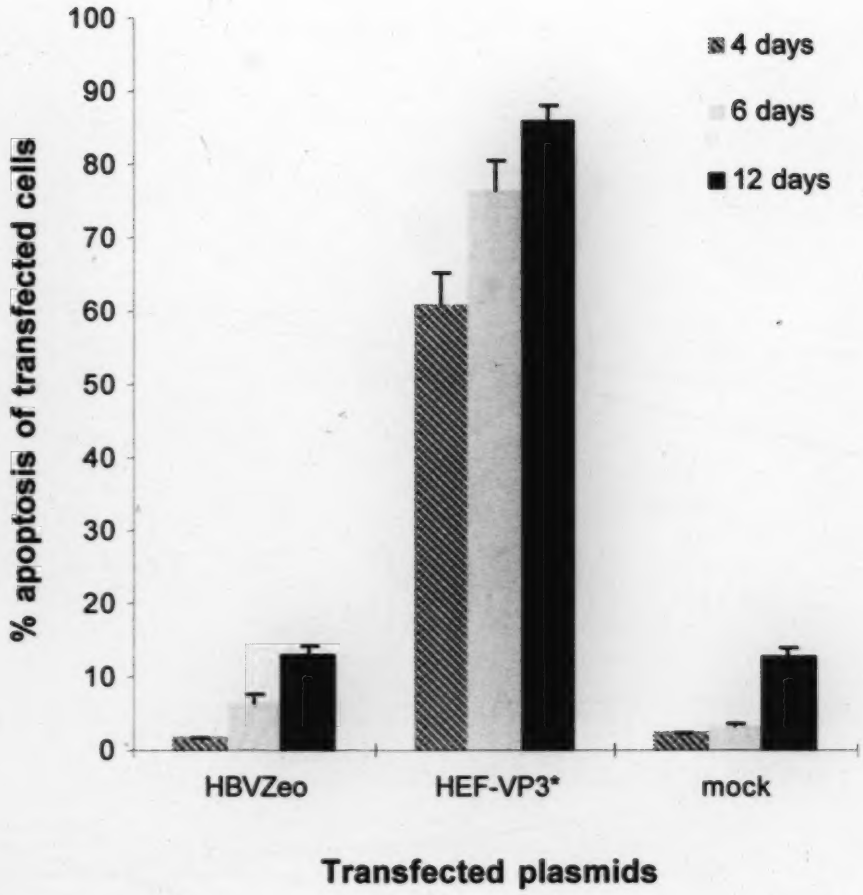
100
90
80
70
60
50
40
30
20
10
0
% apoptosis of transfected cells
4 days
6 days
12 days
HBVZeo
HEF-VP3*
mock
Transfected plasmids

Figure 4.9 Characteristics of HFS apoptosis induced by cycloheximide. CHX-treated and untreated HFS cells were harvested, stained with Hoechst 33258 and examined under a fluorescence microscope at 2 days (**A-C**), 4 days (**D-F**) or 6 days (**G-I**) after incubations. Cellular apoptosis was identified by nuclear condensation or fragmentation (arrow heads). **A**, **D** and **G**, without CHX treatment; **B**, **E** and **H**, 100 μg/ml CHX treatment; **C**, **F** and **I**, 200 μg/ml CHX treatment. Original magnification, 200 X.

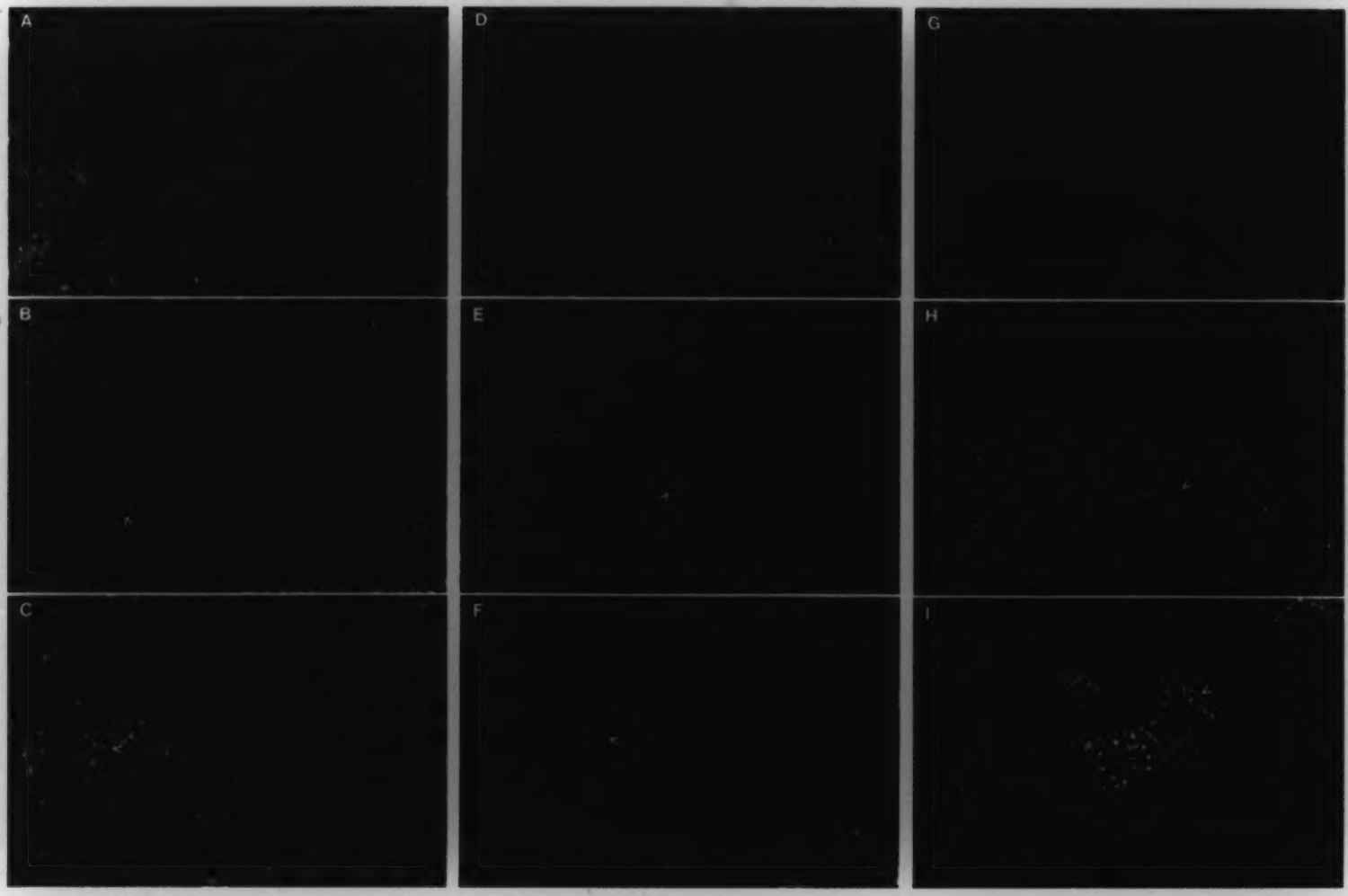
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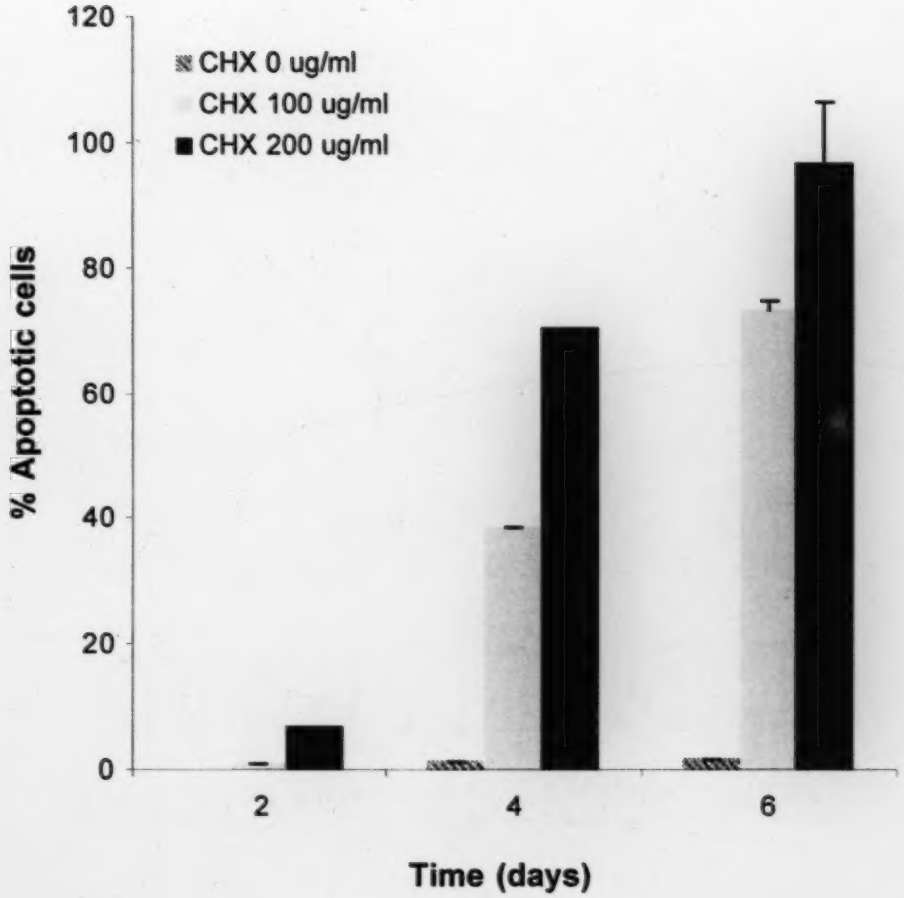
Figure 4.10 Percentages of HFS apoptosis induced by cycloheximide. HFS cells were treated with 100 μg/ml or 200 μg/ml CHX or without CHX for 2, 4 and 6 days. Cellular apoptosis was examined by Hoechst 33258 staining. The apoptotic rates of HFS as percentages of total cells were determined and are presented as averages of three different experiments with standard deviations.


120
100
80
60
40
20
0
% Apoptotic cells
CHX 0 ug/ml
CHX 100 ug/ml
CHX 200 ug/ml
2
4
6
Time (days)

Superfect transfection (Qiagen Inc., Santa Clarita, CA, USA), $CaPO_4$ transfection and the Gene Gun technique. However, the transfection efficiencies obtained from these experiments were very low. Less than 0.001% of cells were transfected, which was not sufficient for further analysis of the CAV-VP3 expression.

According to published reports, however, the CAV-VP3 gene induces apoptosis in transformed cells but not in normal cells.[22,25]

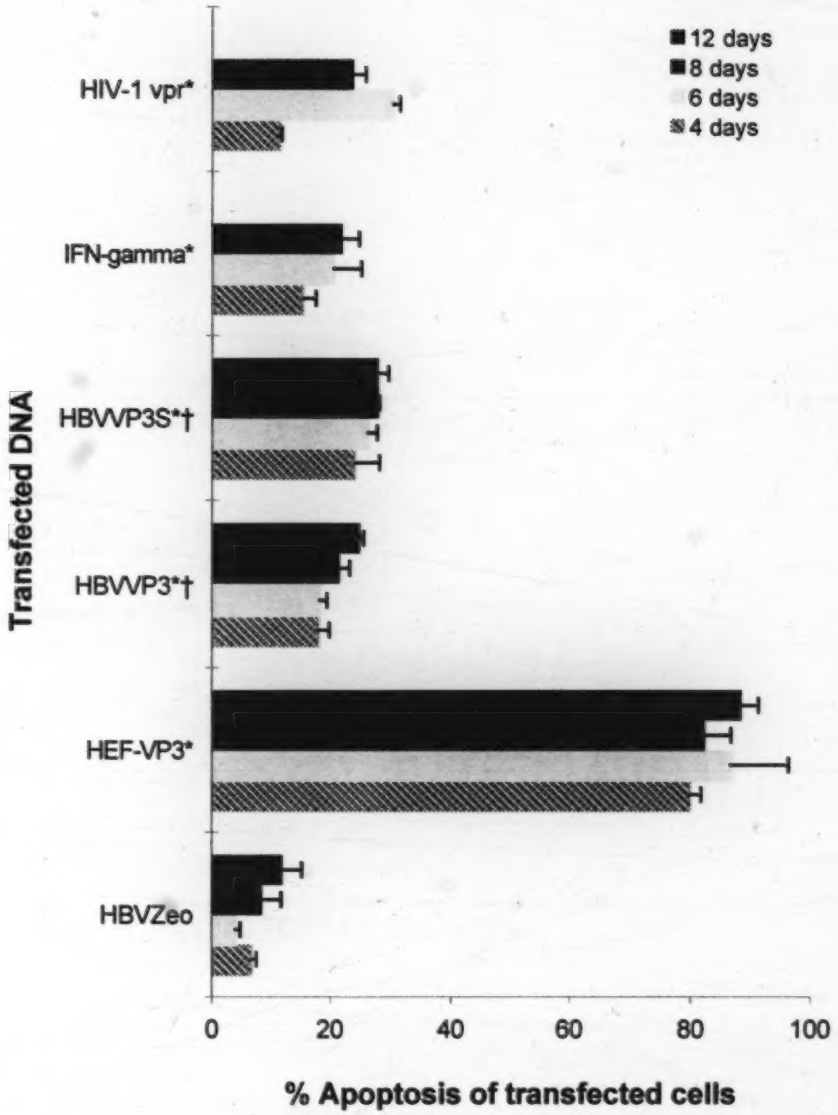
Expression and apoptotic induction of CAV-VP3 by HBV vectors in HepG2

To determine whether the CAV-VP3 gene could be expressed in the context of HBV vectors, I first investigated by cotransfecting HepG2 cells with the GFP expressing plasmid and pTHBVVP3-d, pTHBVVP3S-d, pHEF-VP3 (a positive control) or pTHBVZeo-d (a negative control). Cotransfection of the cells with the plasmids expressing IFN-γ and HIV-1 vpr were also included as positive controls since IFN-γ and HIV-1 vpr have been shown to induce apoptosis in some human transformed cell types.[31] Transfected cells and cellular apoptosis were identified through the expression of GFP and nuclear staining with Hoechst 33258, respectively. Different sets of transfection experiments were performed using Lipofectin (one set of experiment) and the Gene Gun technique (two individual sets of experiments).

As described before, the transfection efficiency obtained from the two different techniques was not significantly different. The percentages of cells induced to undergo apoptosis in the same sample transfected by these different methods were also not different. However, cells transfected by Gene Gun seemed to express stronger signal of GFP than those transfected by Lipofectin.

The apoptotic rates of the HBVVP3- and HBVVP3S-cotransfected cells detected at 4, 6, 8 and 12 days post-transfection were significantly higher than those detected in the negative controls (Figure 4.11). The apoptotic activities of the HBVVP3S-cotransfected

Figure 4.11 Percentages of HepG2 cells exhibiting apoptosis induced by HBVVP3 and HBVVP3S. HepG2 cells were cotransfected with pHEF-GFP and pTHBVVP3-d (HBVVP3), pTHBVVP3S-d (HBVVP3S), pHEF-VP3 (HEF-VP3), pHEF-IFN-γ (IFN-γ), pHEF-vpr (HIV-1 vpr) or pTHBVZeo-d (HBVZeo) and analyzed for apoptotic induction using a fluorescence microscopic assay as described in the text. Percentages of transfected cells showing apoptotic induction at 4, 6, 8 and 12 days post-transfection are presented as averages of three individual experiments with standard deviations. The apoptotic rates induced by IFN-γ and HIV-1 vpr at 12 days post-transfection were not determined. *The percentages of apoptosis of cell cotransfected with these genes were determined to be statistically significant compared with the negative control (ANOVA, $p < 0.01$). †The difference in the percentages of apoptosis of cells transfected with HBVVP3 and with HBVVP3S was statistically significant (ANOVA, $p < 0.01$).


12 days
8 days
6 days
4 days
HIV-1 vpr*
IFN-gamma*
HBVVP3S*†
HBVVP3*†
HEF-VP3*
HBVZeo
Transfected DNA
0
20
40
60
80
100
% Apoptosis of transfected cells

cells were also higher than those induced by IFN-γ and HIV-1 vpr, which exhibited about the same activities as those conferred by HBVVP3. Statistical analyses also indicated that the apoptosis induced in the HBVVP3-cotransfected cells was significantly lower than that induced by HBVVP3S ($p < 0.01$). Increases in the incubation period did not have great effect on the apoptotic rates of the HBVVP3S-cotransfected cells but had significant influence on the rates of the cells transfected with HBVVP3 ($p < 0.05$). The characteristics of cellular apoptosis induced in cells cotransfected with HBVVP3 and HBVVP3S at different periods of time are shown in Figure 4.12 and 4.13, respectively.

To directly verify the findings that the CAV-VP3 protein expressed from the HBV replicative and nonreplicative vectors induced apoptosis in human hepatoma cells, HepG2 cells grown on coverslips were transfected with pTHBVVP3-d, pTHBVVP3S-d, pHEF-VP3 (a positive control) or pTHBVZeo-d (a negative control). Indirect immunofluorescence analyses using mouse ascites anti CAV-VP3 monoclonal antibody were performed to identify the expression of the CAV-VP3 protein from transfected cells. Concomitantly, apoptotic induction in the CAV-VP3 expressing cells was examined by Hoechst 33258 staining of the cellular nuclei. The expression and apoptosis inducing ability of the CAV-VP3 protein in the positive plasmid clearly verified that the CAV-VP3 protein induced apoptosis in HepG2 cells, confirming the data obtained in the above experiments (Figure 4.14, A-C). Moreover, the results showed that the CAV-VP3 protein could be expressed by both HBV replicative and nonreplicative vectors and appeared to trigger the transfected cells to undergo apoptosis as detected by nuclear fragmentation (Figure 4.14, G-I and Figure 4.14, D-F, respectively). The CAV-VP3 protein expressed from both HBV replicative and nonreplicative constructs seemed to be located in both the nucleus and cytoplasm.

However, it was observed that the number of cells expressing the CAV-VP3 protein in the samples transfected with HBVVP3 and HBVVP3S was significantly lower than the positive controls (pHEF-VP3-transfected cells) with similar levels of transfection

efficiency. This observation corresponded to the data obtained in Figure 4.11 which showed that the apoptotic rates of cells cotransfected with HBVVP3 and HBVVP3S were less than those of the positive control. These results indicated the stronger expression of the protein by the HEF 1α gene promoter compared with the HBV promoter.

These data did illustrate clearly that the CAV-VP3 gene was expressed from both HBV replicative and nonreplicative vectors and could induce apoptosis in HepG2 cells. HepG2 apoptosis induced by the CAV-VP3 single protein expressed from the HBV nonreplicative construct was higher than that induced by the fusion protein.

Figure 4.12 Characteristics of HepG2 apoptosis induced by HBVVP3. HepG2 cells were cotransfected with pTHBVVP3-d and pHEF-GFP and examined for apoptotic induction at 4 days (**A-C**), 6 days (**D-F**) and 12 days (**G-I**) post-transfection using a fluorescence microscope. Transfected cells were detected by GFP expression using a green FITC filter (**A**, **D** and **G**). The nuclei of these cells were identified concomitantly by Hoechst 33258 staining using a blue DAPI filter (**B**, **E** and **H**). Apoptosis induced in the transfected cells was identified as fragmentation of the cellular nuclei. Double exposure of these cells with GFP expression and Hoechst staining are also shown (**C**, **F** and **I**). Original magnification, 1000 X.

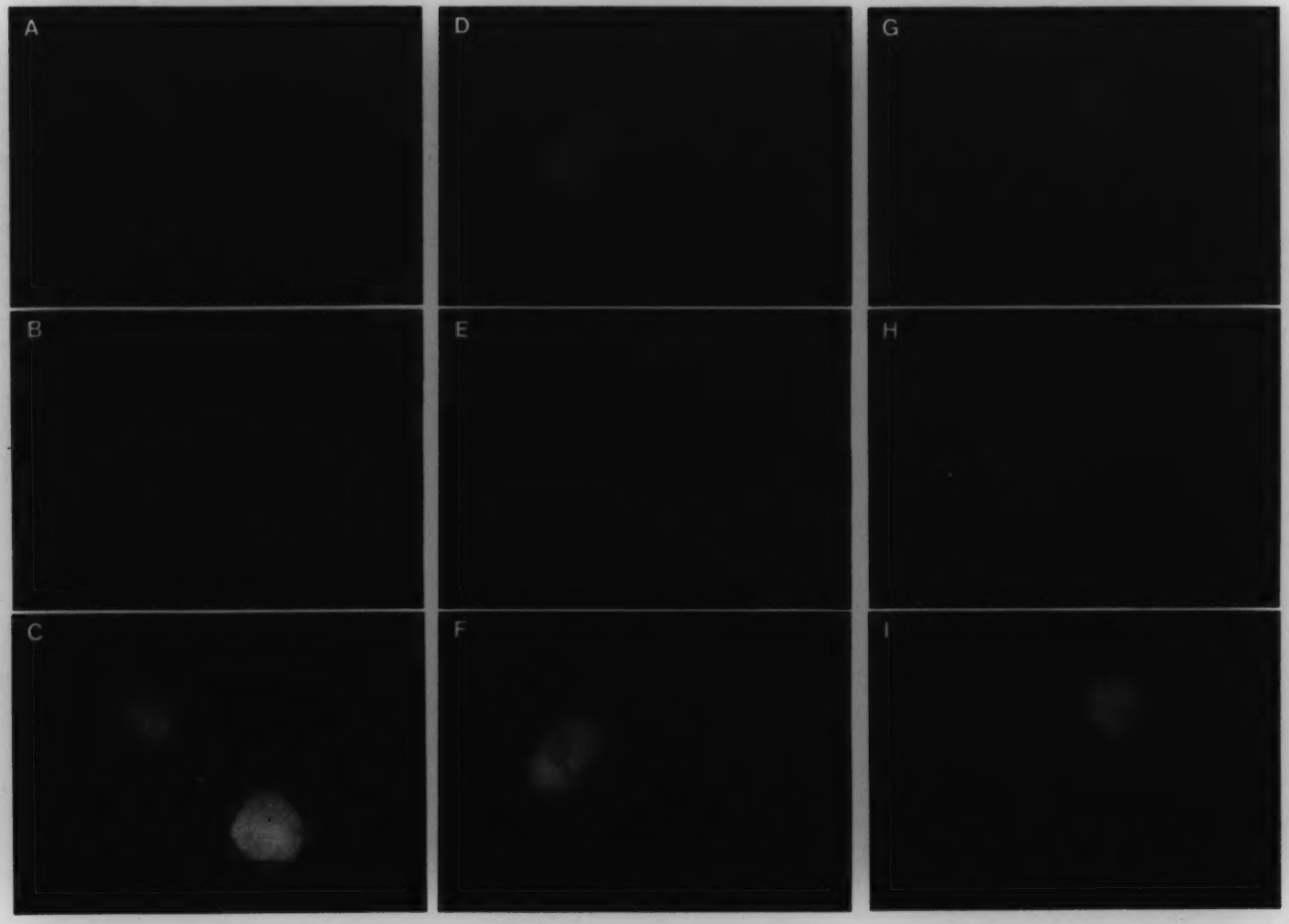

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Figure 4.13 Characteristics of HepG2 apoptosis induced by HBVVP3S. HepG2 cells were cotransfected with pTHBVVP3S-d and pHEF-GFP and examined for the apoptotic induction at 4 days (**A-C**), 6 days (**D-F**) and 12 days (**G-I**) post-transfection using a fluorescence microscope. Transfected cells were detected by GFP expression using a green FITC filter (**A**, **D** and **G**). The nuclei of these cells were identified simultaneously by Hoechst 33258 staining using a blue DAPI filter (**B**, **E** and **H**). Apoptosis induced in the transfected cells was identified as fragmentation of the cellular nuclei. Double exposure of these cells with GFP expression and Hoechst staining are also shown (**C**, **F** and **I**). Original magnification, 1000 X.

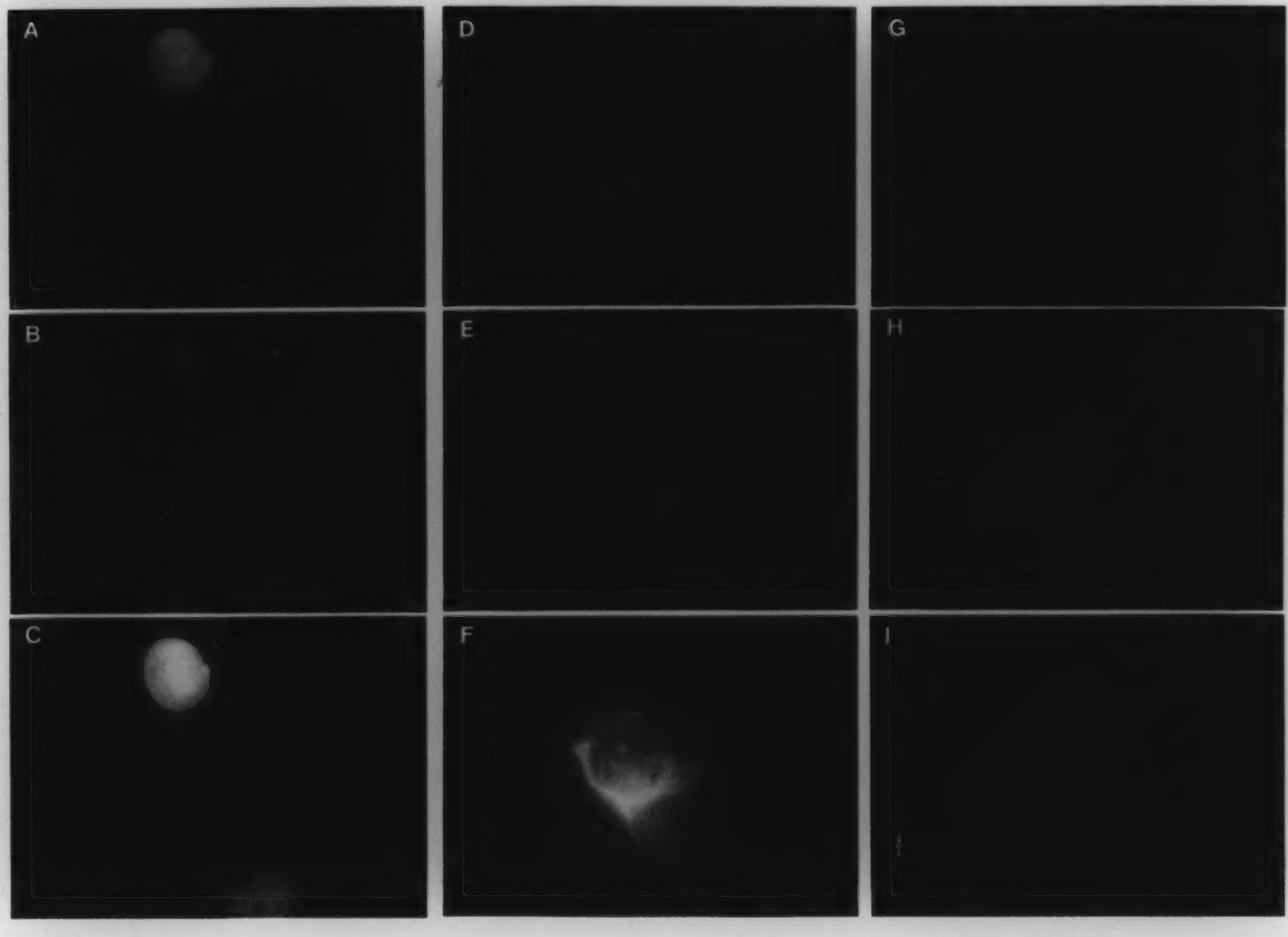
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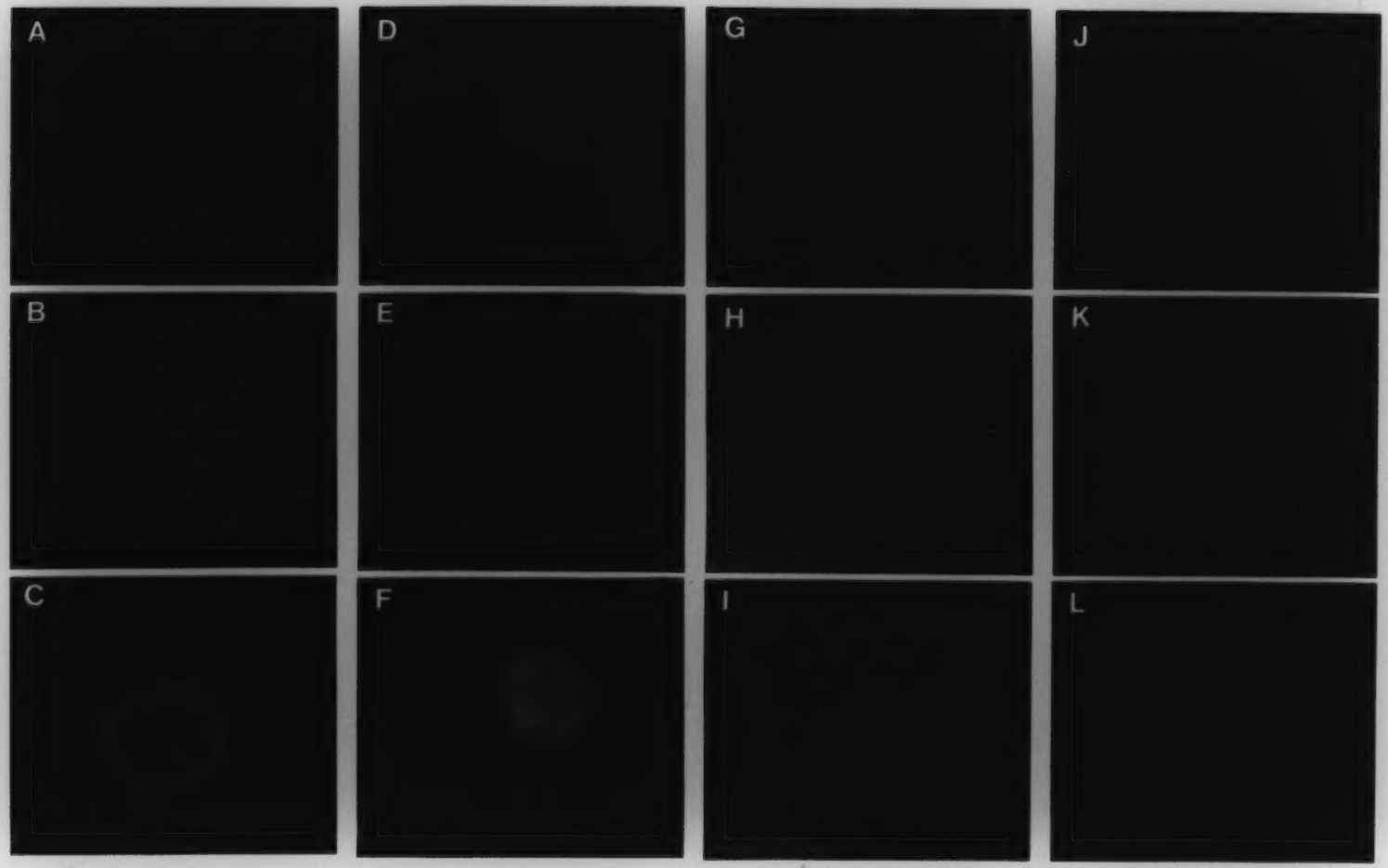
Figure 4.14 Expression of the CAV-VP3 protein from HBV replicative and nonreplicative constructs. HepG2 cells were transfected with pHEF-VP3 (**A-C**), pTHBVVP3S-d (**D-F**), pTHBVVP3-d (**G-I**) and pTHBVZeo-d (**J-L**) used as a negative control. Six days after transfection, indirect immunofluorescence assays were performed to directly identify CAV-VP3 expression using mouse anti CAV-VP3 monoclonal antibody and fluorescein-conjugated anti-mouse IgG. The expressed CAV-VP3 protein was visualized using a green FITC filter (**A**, **D** and **G**) but not in the negative control (**J**). Apoptosis induced in the CAV-VP3 expressing cells was identified as nuclear fragmentation by the Hoechst staining (**B**, **E** and **H**). Double exposure of these cells are shown in **C**, **F** and **I**, respectively. Original magnification, 1000 X.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IV. Discussion

Two major conclusions can be drawn from this study. First, manipulated HBV vectors, both replication-competent and nonreplication-competent types, can be employed for the expression of the potential antitumor CAV-VP3 gene. Second, the CAV-VP3 can induce apoptosis in human hepatoblastoma cells as illustrated by the expression of the gene by both the HEF 1α and HBV promoters.

This study has shown that CAV-VP3 was expressed from both HBV replicative (HBVVP3) and nonreplicative (HBVVP3S) vectors in human hepatoma cells. The apoptotic activity induced by the CAV-VP3 protein expressed from the HBV vectors is significant compared to the activities conferred by other apoptotic agents, IFN-γ and HIV-1 vpr. However, the gene expression controlled by the HBV promoter is lower than that driven by the HEF 1α gene promoter. This is not unexpected since the HEF 1α gene promoter is considered a very strong regulatory element.[32] This promoter is constitutively active in all or most mammalian cell types since it regulates the expression of the protein essentially involved in cellular protein synthesis.[33] The level of gene expression by HBV vectors may be sufficient for therapeutic use *in vivo*; however, further studies are required.

This study has indicated that the C-terminus fusion form of the CAV-VP3 protein expressed from the HBV replicative construct (HBVVP3) is still active and induces apoptosis in human hepatoma cells. However, the activity of the fusion protein seems to be retarded and/or reduced compared with that of the single protein expressed from the HBV nonreplicative construct (HBVVP3S). An explanation for this observation may be that change in the structural conformation of the fusion protein may affect a functional domain of the protein thus causing reduction or retardation of the activity. This hypothesis is supported by recent studies showing that the C-terminus truncated form of this protein exhibits delayed and reduced apoptotic activity compared with that of the full-length protein.[23,24] It has been reported that nuclear localization of this CAV-VP3 protein is

required for optimal apoptotic induction.[23,25] Therefore, one might also hypothesize that the structural conformation of the fusion protein might hamper its migration to the nucleus, where it might need to be located in order to function. However, our data do not seem to be in agreement with this latter speculation since the CAV-VP3 protein expressed from the HBV replicative construct appear to be localized in both the nucleus and cytoplasm.

We have also found that the two different methods for transfection, Lipofectin and Gene Gun, do not have a significant effect on transfection efficiencies or apoptotic frequencies. However, it appears that the Gene Gun approach may introduce more DNA copies into the cells than the liposome transfection method (A. Gainer, personal communication) as evidenced by stronger signal of the GFP expression in cells transfected using the Gene Gun.

One critical consideration in developing gene delivery vectors, particularly for expression of toxic or suicide gene(s) in cancer gene therapy, is that the expression of that gene(s) must be restricted to the tumor cells. For gene therapy of liver tumors, therefore, specific gene transfer to and expression in hepatoma cells is essential. Cellular liver-specific regulatory elements, such as the α-fetoprotein promoter, have been incorporated into adenoviral and retroviral vectors to drive gene expression specifically in hepatoma cells.[6,34] However, employing these liver specific regulatory systems may not solve the problem of the specificity of gene targeting since these viral systems possess wide host ranges. Furthermore, since liver tissues are not their specific natural host, poor transduction ability of these vectors in hepatocytes or hepatoma cells may be another obstacle; one that has arisen with the use of adenoviral vectors.[6,7] HBV vectors, therefore, would be better gene delivery systems for hepatic cells since the transduction and transcription of HBV is primarily restricted to the liver. In Chapter 2, I have demonstrated that the expression of a transgene, the HIV-1 *tat*, by an HBV vector was significantly higher in human hepatoma cells than in nonhuman or nonhepatic transformed cells. However, due to the lack of an *in vitro* system and of an accessible *in vivo* model for

testing HBV infectivity, the transduction ability of HBV vector particles has not yet been determined and is still under investigation.

In conclusion, this study presents another approach for gene therapy for liver malignancies using HBV vectors for gene transfer. The data suggests that an HBV nonreplicative type of vector is a better candidate than a replication-competent vector for several reasons. First, with respect to concern of safety, an HBV nonreplicative vector would be more favorable for therapeutic use. Other viral vector systems including retroviral, adenoviral or herpes simplex viral vectors, are also replication-deficient.[3,35,36] Second, an HBV nonreplicative vector expresses a transgene in a single form to retain its normal activity; therefore, the structural constraint on the functional activity possibly associated with a fusion protein expressed from an HBV replicative vector would be eliminated. Third, the size of the gene to be delivered by an HBV nonreplicative vector would not be as limited. Since replication of this vector could be *trans*-complemented,[12] a large portion of the HBV genome could be replaced with a foreign insert. As a consequence, an HBV nonreplicative vector could also be exploited for transferring a large suicide gene, such as a herpes simplex virus thymidine kinase gene, for cancer gene therapy. The expression of this gene in hepatocarcinoma cells may be sufficient to confer "bystander toxic effect" to the whole tumor tissues.[37,38]

While these results are encouraging, further studies and modification of HBV vectors must be pursued so that the goal of developing HBV as highly efficacious gene delivery systems for therapy of liver diseases and malignancies can be achieved.

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CHAPTER 5

An *in vitro* assay for hepatitis B virus infectivity

I. Introduction

Hepatitis B virus (HBV), like other members of the hepadnaviridae family, is characterized by distinct hepatotropism and a narrow host range. Due to its medical importance as a common cause of acute and chronic hepatitis in humans, the life cycle and pathogenicity of HBV have been studied extensively. Much progress in understanding the regulating mechanism of its replication at the molecular level has been made in recent years.[1] Most of the studies on HBV are based on *in vitro* transfection of human hepatoma cells, particularly HepG2 cells.[2-4] However, the early events of the HBV life cycle, for instance viral entry, are mostly extrapolated from *in vitro* and *in vivo* models of infection of primary duck hepatocytes with duck hepatitis B virus.[5-7] Neither a practical animal model nor an *in vitro* culture system which supports productive HBV infection have been available. Primary human hepatocytes have been shown to be susceptible to HBV infection *in vitro*.[8-10] However, infection of these hepatocytes is not always successful since the infection efficiency varies greatly depending on individual cell origins.[8] Moreover treatment of primary hepatocytes with chemical reagents, such as dimethyl sulfoxide[8,10] or polyethylene glycol,[9] is required for the enhancement and reproducibility of infection. These systems, therefore, may not be representative of a natural HBV infection.

There is a need for an *in vitro* system which supports HBV infection and replication that closely mimicks the natural event. Such a system is essential for testing infectivity of HBV or HBV recombinant particles and could serve as a good model for studying the cellular uptake of HBV. Many laboratories use the HepG2 cell line since an efficient transfection system has been established using this cell line for the study of HBV

replication. However, HepG2 cells do not seem to be susceptible to HBV infection in the hands of most researchers, although these cells can bind HBV.[11,12] It has been speculated that HepG2 cells may not efficiently support HBV internalization. Nevertheless, one laboratory has reported infection of HepG2 cells with HBV-positive serum.[13] Recently, Lu and colleagues[14] have established an *in vitro* infection assay for HBV in HepG2 cells. Their study was based on the hypothesis that proteolytic processing of viral envelope proteins may be necessary for HBV internalization as is required by other enveloped virus for cellular entry.[15-17] Such a process allows fusion of the viral envelope directly with a cellular membrane or with endosomal membranes after endocytosis.[16,18] Therefore, the nonsusceptibility of HepG2 cells to HBV infection may be due to the inability to present the fusion domain of HBV surface proteins to the target cells. These authors demonstrated that HBV treated with staphylococcal V8 protease, which causes cleavage of the preS1 and preS2 domains to expose the putative fusion domain on the S region of the HBV envelope, can infect HepG2 cells at pH 5.5. HBV replication in the infected cells is demonstrated by the appearance of covalent closed circular DNA (cccDNA), viral RNA, viral core and preS2 antigens.

In this study, we employed the infection technique reported by Lu and colleagues to determine the infectivity of HBV recombinant particles produced from HBV vectors manipulated as described in the previous chapters. To function as efficient viral vectors, HBV recombinant particles must be able to transduce a transgene for specific expression in target liver cells. HBVtat recombinant particles produced from an HBV replicative vector carrying the human immunodeficiency virus type 1 (HIV-1) *tat* gene[19] were used for the study. Prior to testing for HBVtat infectivity, the infection protocol was first examined to see whether or not this protocol was practical and reproducible. The infection study was performed in hepatoma cell cultures, HepG2 and Huh-7, using HBV either untreated or treated with staphylococcal V8 protease.

II. Materials and Methods

Plasmid construction

pTHBV-d and pTHBVT-d are replication-competent constructs which carry two copies of the full length HBV (*Eco*RI-*Eco*RI) and HBVtat (*Eco*RI-*Eco*RI) sequences, respectively, ligated in a head to tail tandem configuration as described in Chapter 2. pTHBV contains the full length HBV genome inserted into the *Eco*RI cloning site of pT7T3 18U (Pharmacia Biotech, Uppsala, Sweden).

Cell culture and culture media

Human hepatoblastoma (HepG2) and hepatoma cells (Huh-7) were cultured and maintained at 37°C in 5% CO_2 in Auto-Pow MEM Eagle (modified) medium (ICN Biomedicals Inc., Costa Mesa, CA, USA) supplemented with 10 mM sodium bicarbonate, 2 mM L-glutamate, 10% fetal bovine serum, 50 units/ml penicillin G sodium, 0.01 mg/ml streptomycin and 50 units/ml nystatin.

Primary human hepatocytes (hNHeps™) grown in matrigel® in 6-well tissue culture dishes were purchased from Clonetics Corporation (San Diego, CA, USA). The cells were maintained at 37°C in 5% CO_2 in HMM™ (Clonetics Corporation) supplemented with 10^{-7} M insulin, 10^{-7} M dexamethasone, 50 µg/ml gentamicin and 50 ng/ml amphotericin B.

The pH 5.5 medium used for infection assay was Auto-Pow MEM Eagle (modified) medium supplemented with 2 mM L-glutamate, 10% fetal bovine serum, 50 units/ml penicillin G sodium, 0.01 mg/ml streptomycin and 50 units/ml nystatin and adjusted to pH 5.5 with 1 M 2-[N-Morpholino] ethanesulfonic acid (MES).

Transfection

Transfections of HepG2 cells were performed in 60-mm tissue culture dishes using Lipofectin (GIBCO BRL Life Technologies, Gaithersburg, MD, USA) as recommended by the manufacturer with modifications as described in Chapter 2.

To assess transfection efficiency, transfections were performed in the presence of human growth hormone plasmid pXGH5 (Nichols Institute Diagnostics, San Juan, Capistrano, CA, USA). Secreted human growth hormone was quantitated by radioimmunoassay using a HGH-TGES transient gene expression kit (Nichols Institute Diagnostics).

HBeAg and HBsAg expressed by HBV or HBVtat transfected cells were determined by a Microparticle Enzyme Immunoassay (MEIA) (Abbott Laboratories, Abbott Park, IL, USA).

Isolation of viruses for infection assay

HBV and HBVtat viral particles used for the infection assays were obtained from HepG2 cells transfected with pTHBV-d and pTHBVT-d, respectively. HBsAg and HBeAg were used as viral markers. Six days after transfection, the culture media from the transfected cells were collected and centrifuged in a Sorvall RT6000B Refrigerated Centrifuge (Dupont, Mississauga, ON, Canada) at 2500 g for 10 min to remove cellular debris. The extracellular viral particles were pelleted over a 30% sucrose cushion in 50 mM Tris (pH 8.0), 150 mM NaCl and 10 mM EDTA solution using an ultracentrifuge SW41 rotor at 150 000 g and 4°C for 7-20 hr. The pellets were resuspended in 1 ml of 50 mM Tris (pH 7.5), 150 mM NaCl and 10 mM EDTA. To remove contaminating DNA or RNA, 6 mM MgCl2, 100 µg/ml DNase I and 10 µg/ml RNase A were added to the suspension and incubated at 37°C for 30 min. The digested solution was centrifuged at 4°C using microcentrifugation. The supernatants were transferred to fresh Eppendorf tubes. The virus particles were

precipitated by addition of 300 µl of 26% polyethylene glycol (PEG) 8000, 1.4 M NaCl, and 25 mM EDTA and incubation at 4°C for at least 1 hr. After centrifugation, the pellets were resuspended in appropriate solutions. For untreated HBV or HBVtat, the pellets were suspended in phosphate-buffered saline solution (PBS) and aliquots were kept at -80°C for further use in infection experiments. For treatment with staphylococcal V8 protease (V8), the pellets were resuspended 0.05 M potassium phosphate buffer (pH 7.4).

V8 protease treatment of viruses

The viral suspension was digested with 1.2 mg/ml staphylococcal V8 protease (Endoproteinase Glu-C; Boehringer Mannhiem, Laval, Quebec, Canada) in 0.05 M potassium phosphate buffer (pH 7.4) at 37°C overnight. The protease enzyme was removed by ultracentrifugation over a 20% sucrose cushion using a SW60 rotor at 300 000 g at 10°C for 8 hr. The pellets were resuspended in appropriate volumes of PBS. Aliquots of V8-treated viruses were kept at -80°C for infection studies.

Quantitation of viruses by dot blot analysis

Dot blot hybridization was performed for quantitation of HBV or HBVtat virions. pTHBV with known concentrations was used as a reference standard.

Different dilution of untreated and V8-treated viruses were dotted on pre-wetted Hybond-N membrane (Amersham Life Science, Oakville, ON, Canada) using a Bio-Rad dot blotting apparatus. The DNA was denatured on filter paper soaked with 1.5 M NaCl, 0.5 M NaOH for 30 min and neutralized with 1.5 M NaCl, 1M Tris (pH 7.4) for 30 min. The membrane was then blotted dry and cross-linked using an ultraviolet light for 2-3 min. Prehybridization and hybridization were then carried out using a standard method as used for Southern blot analysis.[20] ^{32}P-labeled HBV DNA monomer was used as a probe. Dot blot analysis of a series of pTHBV at different concentrations, 2 ng, 1 ng, 500 pg, 100 pg

and 50 pg, was performed on the same membrane as the samples. The relative amounts of hybridized DNA were determined using a phosphoimager (BAS1000, Fuji, Kanagawa, Japan).

The number of HBV or HBVtat virions per ml was calculated using a linear plot of a dilution series of the reference standard. It was assumed that an HBV monomer is equivalent to one virion (or molecule) of HBV or HBVtat. The number of virions was calculated based on the fact that one gram molecular weight of a substance contains 6.02×10^{23} molecules.

HBV and HBVtat Infection

Infectivity assays of HBV and HBVtat were performed in HepG2, Huh-7 and hNHeps™ using the procedure described by Lu *et. al.*[14] with some modifications. HepG2 and Huh-7 cells were seeded at about 70-80% confluent in 6-well tissue culture dishes or on poly L-lysine coated coverslips in 24-well tissue culture plates. Culture media were changed everyday before infection. Infection experiments were performed 48 hr after seeding, when cells had reached 90-100% confluent. Purchased primary human hepatocytes (hNHeps™) grown in 6-well tissue culture dishes were shipped at ambient temperature 48 hr after being plated. Upon receipt, hNHeps™ were incubated at 37°C in 5% CO_2 for 48 hr prior to performing an infection assay. The cells were about 80% confluent.

Cells were washed briefly and fed with prewarmed pH 5.5 media (0.2 ml per well of 24-well tissue culture plates or 1 ml per well of 6-well tissue culture dishes). An aliquot of viruses, thawed at 4°C, was added to the cells at approximately 10^7 virions per ml culture medium, unless otherwise stated. To distribute viruses evenly, the culture plates were shaken gently. The cells were incubated at 37°C in 5% CO_2 for 12-15 hr. Cells should not be incubated in the pH 5.5 media for longer than 15 hr due to the toxicity of the acidic pH. The culture media containing viruses were removed and unabsorbed viruses were removed

by gently washing twice with pH 5.5 media, twice with PBS and once with normal media. Cells were then fed with normal media and incubated at 37°C in 5% CO_2, the beginning of the incubation period being designated as time zero. Culture media were changed every 2 days.

Detection of infected cells

Infectivity of viruses was determined by immunochemical staining or immunofluorescence assay to detect HBcAg and/or HBsAg expressed from infected cells.

Immunochemical staining for HBcAg

Adherent infected cells were washed three times with PBS and fixed in methanol/acetone (1:1) at 20°C for 2-3 min. After removal of the fixing solution, cells were again washed three times with PBS and incubated with freshly prepared 0.5% H_2O_2 diluted in Tris-Buffered Saline (TBS) (100 mM Tris.Cl, pH 7.5, 0.9% (w/v) NaCl) at room temperature for 10-15 min to neutralize cellular peroxidases. For the assay in primary human hepatocytes, cells were incubated with freshly prepared 1% of the H_2O_2 solution for 15-30 min to neutralize cellular peroxidases. Cells were washed four times with PBS and further incubated in a blocking solution (20% FBS, 0.5% Tween in PBS) at room temperature for 15 min. After removal of the blocking solution, cells were incubated in a 1:500 dilution of rabbit anti-HBcAg polyclonal antibody (Dako, Carpinteria, CA, USA) in the blocking solution at room temperature for 1 hr with gentle shaking. Cells were washed three times with 0.3% Tween 20 in TBS with shaking for 5 min per each wash. A 1:200 dilution of normal goat sera in the blocking solution was added to each sample and cells were incubated at room temperature for 30 min with gentle shaking. Cells were washed once with PBS with 5 min shaking. Biotin-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL, USA) diluted 1:1500 in the blocking solution was added to the cells which were further incubated at room temperature for 30 min with gentle shaking. Cells were

then washed three to four times with 0.3% Tween 20 in TBS with shaking for 5 min per wash. Meanwhile, Avidin-Biotin Complex solution was prepared from an ImmunoPure® Ultra-Sensitive ABC staining kit (Pierce) by mixing one drop of reagent A and B with 2.5 ml of PBS with incubation at room temperature for 30 min. The ABC solution was added to the cells which were further incubated at room temperature for 30 min. Cells were then washed three times with PBS with shaking for 5 min per each wash. Meanwhile, Sigma Fast™ DAB peroxidase substrate solution (Sigma Chemical Co., St. Louis, MO, USA) was prepared by dissolving one urea hydrogen peroxide tablet and one DAB tablet in 4.5 ml of double distilled water (ddH_2O) and 0.5 ml of 3% Nickel chloride solution was added with mixing. The solution was subsequently added to the washed cells. Cells were incubated at room temperature for 1-3 min depending on the strength of the signal. This step was critical since prolonged incubation may result in a high background. Cells were finally rinsed gently with PBS or water for 2-3 min to remove unreacted reagents. The immunochemical staining signal of HBcAg expression was visualized under a light microscope.

Indirect Immunofluorescence for HBcAg and/or HBsAg

Infected cells grown on coverslips were washed twice with IF buffer (2% fetal calf serum and 0.02% sodium azide in PBS) and fixed by incubating with 3% paraformaldehyde in PBS at room temperature for 10 min. Cells were washed three times with IF buffer and permeabilized by incubating with acetone:methanol (1:1) solution at -20°C for 2-3 min. Cells were washed three times with IF buffer. To the washed cells, a 1:100 dilution of rabbit anti-HBcAg polyclonal antibody in IF buffer was added. For double staining of HBcAg and HBsAg, a 1:200 dilution of mouse anti-HBsAg monoclonal antibody purified from H25B10 cells (ATCC No. CRL 8017) in IF buffer was also added. Cells were incubated at room temperature for 1 hr with gentle shaking and subsequently washed four times with IF buffer with shaking for 5 min per wash. A normal goat serum solution in IF

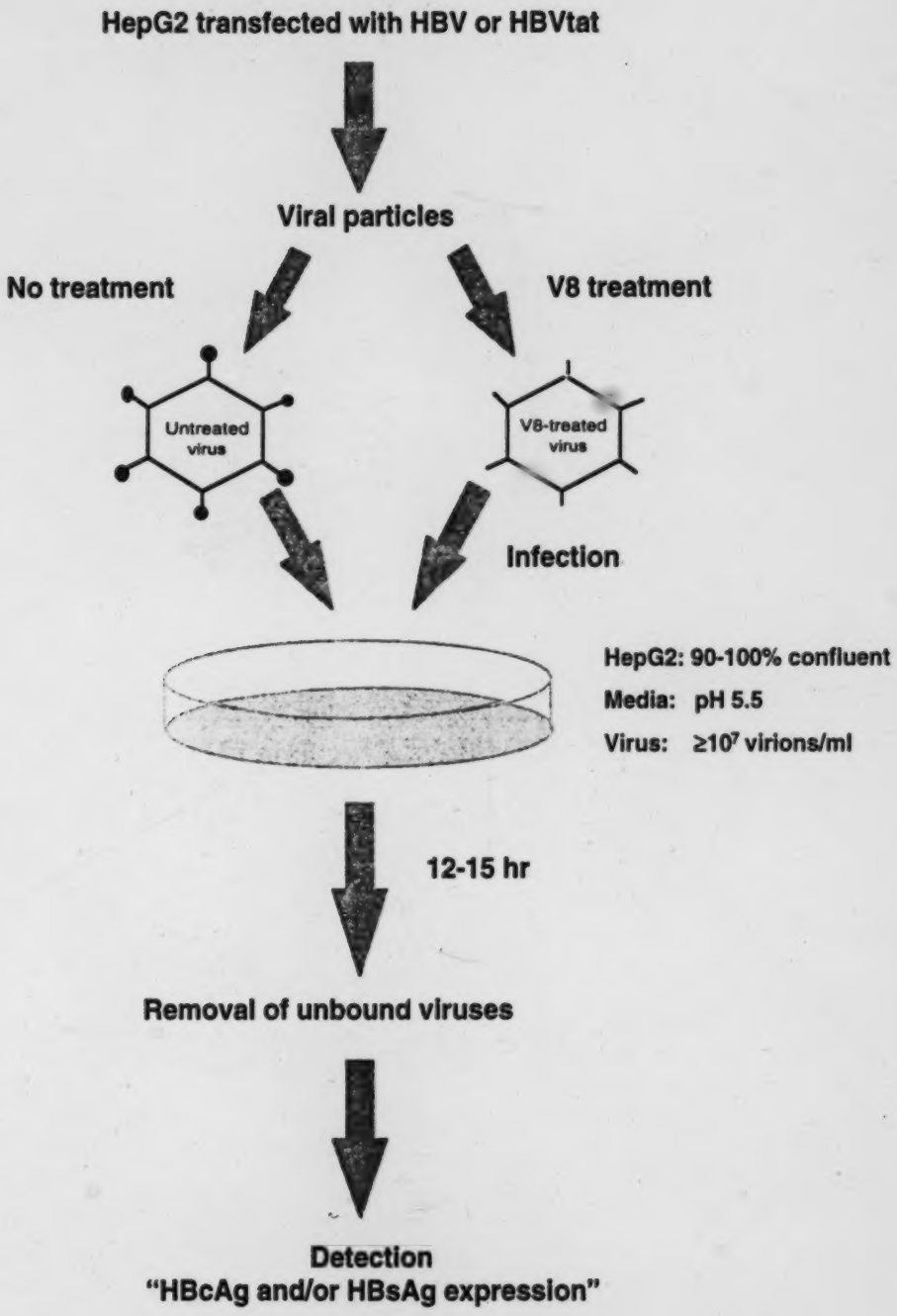
buffer (1:100) was added and cells were further incubated at 37°C for 30 min. After removal of the serum solution, cells were further incubated with a 1:200 dilution of fluorescein-conjugated goat anti-rabbit IgG (Cappel: Organon Teknika Corp., West Chester, PA, USA) in IF buffer at 37°C for 1 hr. For double staining, fluorescein-conjugated goat anti-mouse IgG (Cappel) was also added at a 1:200 dilution. From this step onward, samples were covered with aluminum foil to protect them from light. Cells were washed three times with IF buffer with shaking for 5 min per wash and once briefly with PBS. Cells were covered with SlowFade® Light Antifade solution (Molecular Probes, Eugene, OR, USA), sealed with nail polish and viewed under a fluorescence microscope (Axioskop Zeiss, Germany; Carl Zeiss Canada Ltd., North York, ON, Canada) using a green FITC fluorescence filter.

III. Results

Experimental Strategy

The infection protocol used for this study was adapted from the method described by Lu *et. al.*[14] and outlined as shown in Figure 5.1. To reproduce the experiment of Lu *et. al.*, cells were infected with viruses treated with V8 protease at pH 5.5 media. V8 protease removed the preS1 and preS2 sequences upstream of the putative fusion domain present in the S sequence.[14] The hypothesis underlying this strategy, as proposed by Lu *et. al.*, is that the proteolytic processing of viral envelope proteins and acidic pH may be essential for HBV uptake and may be absent in HepG2 cells. In a second experiment, infection was performed at pH 5.5 using untreated viruses. This experiment was designed to test the hypothesis that the acidic pH which is optimal for uptake of enveloped viruses via pH-dependent endocytolytic pathway[21,22] may be sufficient to support HBV infection in nonsusceptible hepatoma cultures, *e.g.* HepG2 cells. The minimum quantities of viruses used for infection were 10^7 virions per ml of media or 100 m.o.i. (multiplicity of infection) as used in the published report.[14] Although the viral suspension used for infection was a mixture of HBV complete particles (Dane particles) and HBsAg particles, only HBV DNA containing particles were used to determined the number of virions added. Negative controls for these experiments were parallel cultures of HepG2 cells incubated in pH 5.5 media without viruses. Infection efficiencies were initially evaluated by immunochemical staining and immunofluorescence analyses of HBcAg and/or HBsAg expression from infected cells. If these were sufficiently high, infected cells would be further characterized by other analysis methods for viral replication, such as synthesis of cccDNA and intracellular and extracellular HBV DNA.

Figure 5.1 Diagrammatic representation of HBV infection protocol. This was a standard procedure used for infection assays of HBV or HBVtat viruses in all experiments. Viruses were isolated without treatment or with V8 protease treatment as described in Materials and Methods. Since antibodies specific for preS1 and preS2 domains were not available in our laboratory, we assumed that the V8 protease digestion was complete since the treatment was carried out as described by the original paper.[14] Cells were grown in monolayers for about 48 hr to semiconfluent (90% to almost 100% confluent), except for primary human hepatocytes, and incubated with virus at pH 5.5 media. Cells must be healthy so that they are able to survive the 12-15 hr incubation in the acidic media. After incubation, the unadsorbed virions were removed by washing several times with pH 5.5 media, PBS and normal media as described in Materials and Methods. Infection efficiency was determined typically at six days post-infection, unless otherwise stated. Immunochemical staining or immunofluorescence analyses for detection of HBcAg and/or HBsAg expression were used as initial detection methods for infected cells since these methods could detect infection at single cell level.


HepG2 transfected with HBV or HBVtat
Viral particles
No treatment
V8 treatment
Untreated virus
V8-treated virus
Infection
HepG2: 90-100% confluent
Media: pH 5.5
Virus: ≥10⁷ virions/ml
12-15 hr
Removal of unbound viruses
Detection
"HBcAg and/or HBsAg expression"

Infection of HepG2 and Huh-7 with HBV

The first trials were performed in HepG2 cells in order to test the reproducibility of the results described in the paper published by Lu *et. al.*[14] Huh-7 cells were also used to test whether or not this protocol could be applied with other human hepatoma derived cells. Cells were incubated with untreated and V8-treated HBV at an m.o.i. of approximately $5x10^2$ and 10^4 in pH 5.5 media. Six days after infection, the samples were examined for infected cells by detection of HBcAg expression using immunochemical staining. The results revealed that very low numbers of the HepG2 cells incubated with untreated HBV at $5x10^2$ m.o.i. or with V8-treated HBV at $5x10^2$ and 10^4 m.o.i. expressed HBcAg (Table 5.1). About 5-10 positive cells were detected per plate of approximately 10^5 cells examined. Huh-7 cells showed the same number of positive cells expressing HBcAg only if they were incubated with V8-treated HBV at 10^4 m.o.i.(Table 5.1). The peroxidase-HBcAg immunochemical staining of the HepG2 cells infected with untreated and V8-treated HBV at $5x10^2$ m.o.i. are shown in Figure 5.2. Positive Huh-7 and HepG2 cells (infected with 10^4 m.o.i. of V8-treated HBV) are shown in Figures 5.3 and 5.4, respectively. Since the infection efficiency was very low, other detection methods were not used to evaluate infectivity.

Despite the low infection efficiencies, these studies suggested that hepatoma cells, HepG2 and Huh-7, could be infected with untreated and V8-treated HBV at pH 5.5. Increasing the multiplicity of infection did not seem to significantly enhance the infection efficiency, particularly in HepG2 cells .

Infection of human primary hepatocytes with HBV

It might be possible to use primary human hepatocytes to determine of HBV infectivity *in vitro* using the standard protocol. Primary human hepatocytes (hNHeps™) were grown in matrigel® for about 4 days and were about 80% confluent at the time of infection.

Table 5.1 Infection of HepG2 and Huh-7 with untreated or V8-treated HBV

Cells[a]	Viruses			Detection[b]	Results[c]
	type	virions/ml	m.o.i.		
HepG2	untreated HBV	$1.5x10^8$	$5x10^2$	IS	positive (≤ 0.0001%)
	V8-treated HBV	$1.5x10^8$	$5x10^2$	IS	positive (≤ 0.0001%)
	V8-treated HBV	$2.8x10^9$	10^4	IS	positive (≤ 0.0001%)
Huh-7	untreated HBV	$1.5x10^8$	$5x10^2$	IS	negative
	V8-treated HBV	$1.5x10^8$	$5x10^2$	IS	negative
	V8-treated HBV	$2.8x10^9$	10^4	IS	positive (≤ 0.0001%)

[a] Cells were grown in 6-well tissue culture plates with a total number of about $3.0x10^5$ cells per well prior to infection. Infection was performed in 1 ml pH 5.5 media.

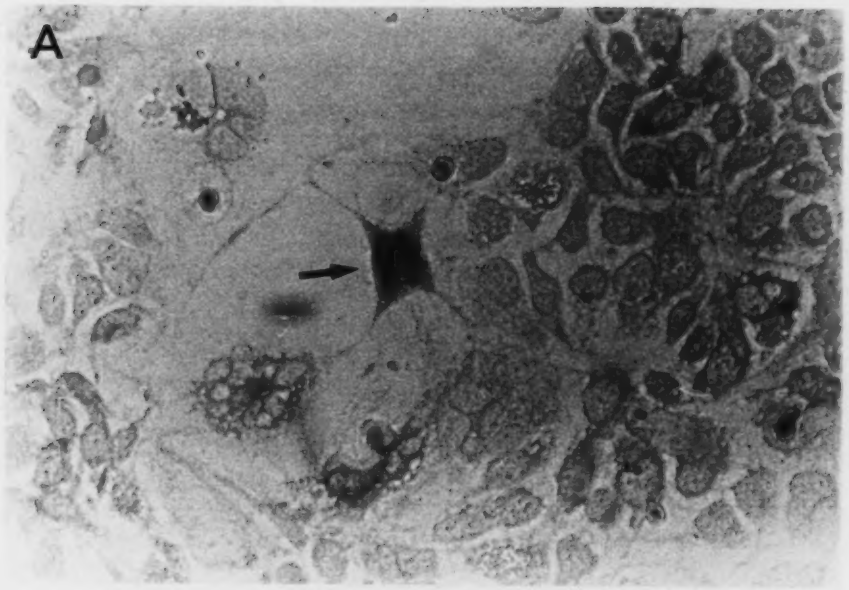
[b] Six days post-infection, infected cells were examined by immunochemical staining (IS) for HBcAg expression as described in Materials and Methods.

[c] Positive results mean that at least one positive cell was detected by visualization under a light microscope. The percentages of positive results were approximate values estimated from a number of positive cells detected per total number of cells used for infection. Negative results represented that no positive cell was visualized after scanning through the whole sample dish.

Figure 5.2 Immunochemical staining of HepG2 infected with untreated and V8-treated HBV at an m.o.i. of $5x10^2$. Cells were incubated with virus as described for Figure 5.1. Six days post-infection, infected cells were identified by immunochemical staining for HBcAg expression. **A**, positive control for the peroxidase-HBcAg immunochemical staining (HBV transfected cells); **B**, negative control; **C**, cells incubated with V8-treated HBV; **D**, cells incubated with untreated HBV. Arrows indicate positive cells expressing HBcAg. Original magnification, 400 X.

A
C
B
D

Figure 5.3 Immunochemical staining of Huh-7 infected with V8-treated HBV at an m.o.i. of 10^4. Infection assays were performed as described for Figure 5.1. Six days post-infection, infected cells were identified by immunochemical staining for HBcAg expression. **A**, cells incubated with V8-treated HBV; **B**, negative control. An arrow indicates a positive cell expressing HBcAg. Original magnification, 400 X.


A

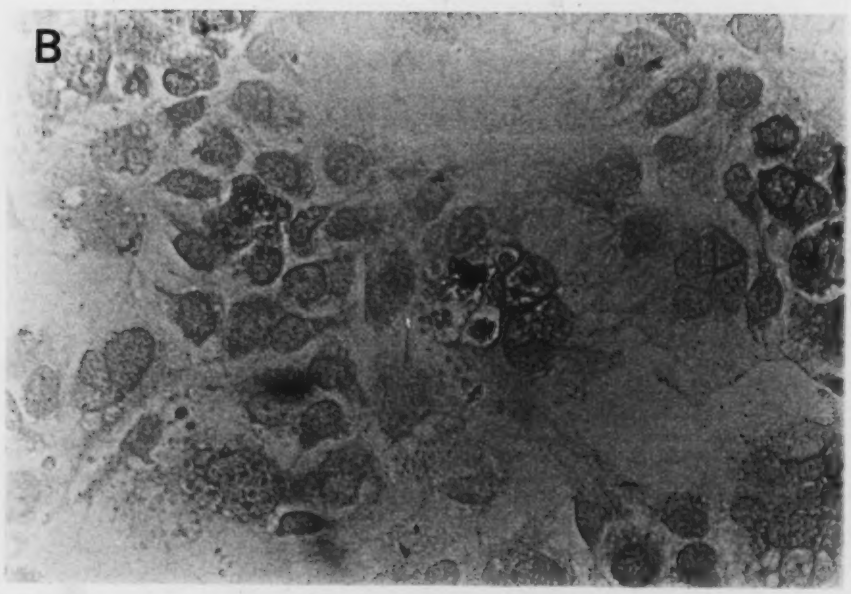
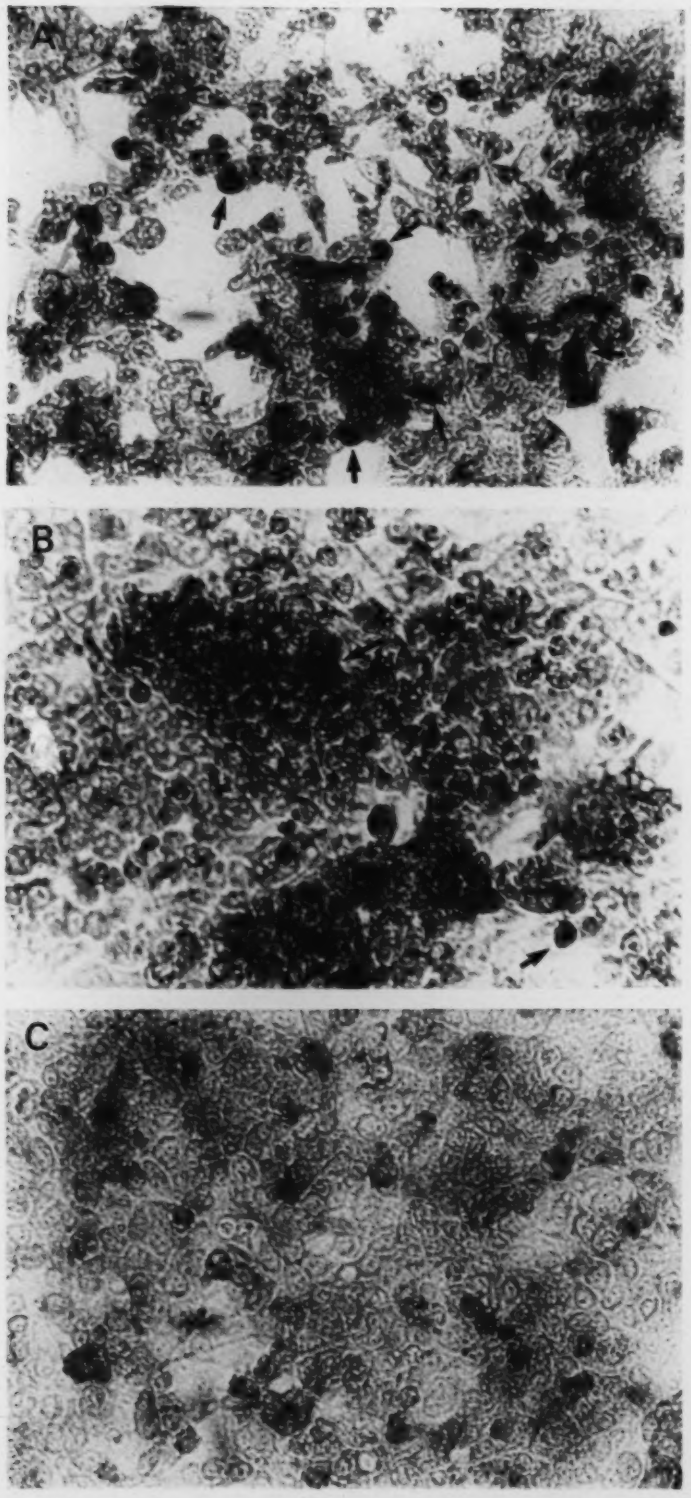

B

Figure 5.4 Immunochemical staining of HepG2 infected with V8-treated HBV at an m.o.i. of 10^4. Infection assays were performed as described for Figure 5.1. Six days post-infection, infected cells were identified by immunochemical staining for HBcAg expression. **A**, positive control for the peroxidase-HBcAg immunochemical staining (HBV transfected cells); **B**, cells incubated with V8-treated HBV; **C**, negative control. Arrows indicate positive cells expressing HBcAg. Original magnification, 400 X.


A
B
C

Infection experiments were performed with untreated and V8-treated HBV at an m.o.i. of 10^3. Six days post-infection, the infection efficiencies were determined using immunochemical staining for HBcAg. This study, however, was not successful. There was no significant difference between the peroxidase-HBcAg immunochemical staining of HBV infected samples and the negative controls. All showed high background due to interference by cellular peroxidase, even though high concentrations of the hydrogen peroxide solution with long incubation period were used for the neutralization step in the immunochemical staining assay. An immunofluorescence assay would be more suitable to determine HBV infected primary hepatocytes, if the infection study in primary human hepatocytes is to be continued. The primary human hepatocytes used in the study may not have been healthy or formed a confluent monolayer since many cells died during the delivery from the supplier at ambient temperature.

Since the results of this study were not encouraging and we did not have a reliable supply of primary human hepatocytes, the study with these cells was discontinued.

Infectivity of HBVtat in HepG2

We were interested in developing an *in vitro* infection assay for HBV in order to use it as a model for examining the infectivity of HBV or HBV recombinant viruses, such as those produced from HBV vectors. In this study, the infectivity of HBVtat recombinant particles produced from a HBV replicative vector carrying the HIV-1 *tat* was determined.

In the first attempt, HepG2 cells were incubated with untreated and V8-treated HBVtat particles at various multiplicity of infection in acidic media. To serve as a control, a parallel set of experiments was also performed with untreated and V8-treated HBV. HepG2 cells used in this study were newly purchased from ATCC (American Type Culture Collection, Rockville, MD, USA) (passage number 76). It was expected that using a lower passage number of HepG2 cells might have a positive impact on the infection efficiency. The

infectivity of the viruses in these samples were determined at six days post-infection using an immunofluorescence assay for HBcAg. The results are shown in Table 5.2. At an m.o.i. of about $5x10^2$, HepG2 cells did not appear to be infected with untreated or V8-treated HBVtat, nor with untreated or V8-treated HBV. With the increased amounts of inoculating virus, however, the results were not conclusive. Although a few positive infected cells were identified, the signals were not as significant as those detected in the positive controls, HBVtat transfected HepG2 cells. We then speculated that cells might be infected by the viruses but expression of the viral genes might be low at the early stages of infection and might not be clearly identified by the detection method. Therefore, prolonged incubation of cells after infection might enhance the detection signal.

Another attempt on infection of HepG2 with HBVtat was subsequently performed. Cells were incubated with untreated and V8-treated HBVtat at various multiplicity of infection in parallel with untreated and V8-treated HBV which served as controls. Infection efficiencies in these samples were determined at seven, ten and thirteen days post-infection using immunofluorescence assay to detect HBcAg and HBsAg. We hypothesized that prolonged incubation of cells and detection for both HBcAg and HBsAg might improve the signals in infected cells. The summary of these results is shown in Table 5.3. At seven days post-infection, few HepG2 cells incubated with untreated and V8-treated HBVtat at an m.o.i. of about 10^3 showed clear positive signals of HBcAg and HBsAg expression (Figure 5.5, C and D). Interestingly, no positive cell was identified in cells incubated with untreated or V8-treated HBV at the same multiplicity of infection (Figure 5.5, E and F). However, a few infected cells with strong signals of HBcAg and HBsAg expression were identified with incubation with untreated HBV at a high multiplicity of infection ($7x10^8$) (Figure 5.6, B). Surprisingly, no clearly positive cells were identified with incubation for longer periods: ten and thirteen days.

Concluding remarks

In our experience, the infection efficiency obtained from this infection protocol was very low; therefore, this procedure may not be practically used as a model for studying HBV infectivity. However, these studies suggested that HepG2 cells are susceptible to both untreated or V8-treated HBV at pH 5.5. These studies also indicate that HBVtat particles are infectious as shown by infection of HepG2 cells with untreated and V8-treated particles.

Table 5.2 Infectivity of HBVtat in HepG2

Cells[a]	Viruses			Detection[b]	Results[c]
	type	virions/ml	m.o.i.		
HepG2	untreated HBV	$2.4x10^8$	$4.7x10^2$	IF	negative
	V8-treated HBV	$2.4x10^8$	$4.7x10^2$	IF	negative
	untreated HBVtat	$2.4x10^8$	$4.7x10^2$	IF	negative
	V8-treated HBVtat	$2.4x10^8$	$4.7x10^2$	IF	negative
HepG2	untreated HBV	$7.1x10^8$	$1.4x10^3$	IF	NC
	V8-treated HBV	$7.1x10^8$	$1.4x10^3$	IF	NC
	untreated HBVtat	$7.1x10^8$	$1.4x10^3$	IF	NC
	V8-treated HBVtat	$7.1x10^8$	$1.4x10^3$	IF	NC
HepG2	untreated HBV	$1.2x10^9$	$2.4x10^3$	IF	NC
	V8-treated HBV	$1.2x10^9$	$2.4x10^3$	IF	NC
	untreated HBVtat	$1.2x10^9$	$2.4x10^3$	IF	NC
	V8-treated HBVtat	$1.2x10^9$	$2.4x10^3$	IF	NC

[a] Cells were grown on poly-L-lysine coated coverslips in 24-well tissue culture plates with a total number of about $1.0x10^5$ cells per well prior to infection. Infection was performed in 0.2 ml pH 5.5 media.

[b] Six days after infection, infected cells were examined by immunofluorescence (IF) for HBcAg expression as described in Materials and Methods.

[c] Negative results mean that no positive signal, when compared with negative controls, was visualized after scanning through the whole sample dish under a light microscope. NC (not conclusive) results represent questionable positive signals detected in a few cells.

Table 5.3 Infectivity of HBVtat in HepG2[a] with prolonged incubation post-infection

Incubation	Viruses			Detection[b]	Results[c]
	type	virions/ml	m.o.i.		
7 days	untreated HBV	$7.0x10^8$	10^3	IF	negative
	untreated HBV	$5.0x10^{14}$	$7x10^8$	IF	positive (<0.0001%)
	V8-treated HBV	$7.0x10^8$	10^3	IF	negative
	untreated HBVtat	$7.0x10^8$	10^3	IF	positive (<0.0001%)
	V8-treated HBVtat	$7.0x10^8$	10^3	IF	positive (<0.0001%)
10 days	untreated HBV	$7.0x10^8$	10^3	IF	negative
	untreated HBV	$5.6x10^9$	$8x10^3$	IF	negative
	V8-treated HBV	$7.0x10^8$	10^3	IF	negative
	V8-treated HBV	$5.6x10^9$	$8x10^3$	IF	negative
	untreated HBVtat	$7.0x10^8$	10^3	IF	negative
	V8-treated HBVtat	$7.0x10^8$	10^3	IF	negative
13 days	untreated HBV	$7.0x10^8$	10^3	IF	negative
	untreated HBV	$5.6x10^9$	$8x10^3$	IF	negative
	untreated HBV	$3.0x10^{14}$	$4x10^8$	IF	negative
	V8-treated HBV	$7.0x10^8$	10^3	IF	negative
	V8-treated HBV	$5.6x10^9$	$8x10^3$	IF	negative
	untreated HBVtat	$7.0x10^8$	10^3	IF	negative
	V8-treated HBVtat	$7.0x10^8$	10^3	IF	negative

[a] Cells were grown on poly-L-lysine coated coverslips in 24-well tissue culture plates to total number of about $1.4x10^5$ cells per well prior to infection. Infection was performed in 0.2 ml pH 5.5 media.

[b] Infected cells were examined by immunofluorescence (IF) for HBcAg and HBsAg expression as described in Materials and Methods.

[c] Positive results mean that at least one positive cell was detected by visualization under a light microscope. The percentages of positive results were approximate values estimated from a number of positive cells detected per total number of cells used for infection. Negative results mean that no positive cell was visualized after scanning through the whole sample dish.

Figure 5.5 Indirect immunofluorescence for HBcAg and HBsAg expression from HepG2 infected with HBV and HBVtat with prolonged incubation post-infection. Cells were incubated with untreated or V8-treated viruses at an m.o.i. of 10^3 as described for Figure 5.1. Seven days post-infection, infected cells were identified by immunofluorescence assays as described in Materials and Methods. **A**, positive control for an indirect immunofluorescence assay (HBVtat transfected cells); **B**, negative control; **C**, cells incubated with untreated HBVtat; **D**, cells incubated with V8-treated HBVtat; **E**, cells incubated with untreated HBV; **F**, cells incubated with V8-treated HBV. Original magnification, 1000 X.

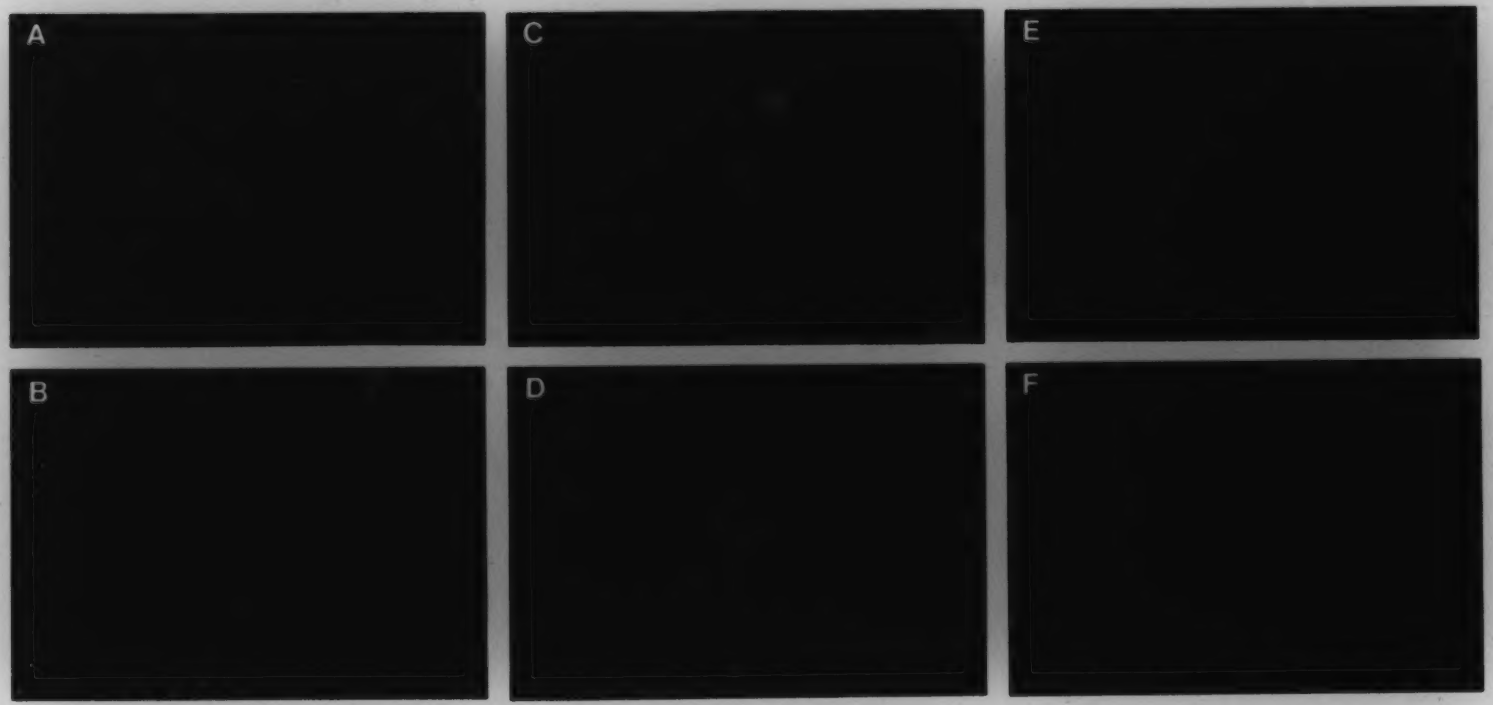

A
C
E
B
D
F

Figure 5.6 Indirect immunofluorescence to detect HBcAg and HBsAg expression from HepG2 infected with untreated HBV at an m.o.i. of $7x10^8$. Infection experiments were carried out as described for Figure 5.5. Seven days post-infection, infected cells were identified by immunofluorescence assays as described in Materials and Methods. **A**, positive control for an indirect immunofluorescence assay (HBVtat transfected cells); **B**, cells incubated with untreated HBV; **C**, negative control. Original magnification, 1000 X.

A

B

C

IV. Discussion

The results of HBV infection in HepG2 cells obtained from our studies are different from the data presented by Lu and colleagues.[14] We have employed their *in vitro* infection protocol for the studies and replicated all the conditions of the infection, except for the viruses and HepG2 cells which come from different sources. In our hands, treatment of HBV with staphylococcal V8 protease does not seem to have a great effect on the susceptibility of HepG2 cells to HBV infection. Very low percentages of HepG2 cells are infected with V8-treated virus. This is significantly different from the results in the published report which stated that 10-30% of HepG2 infected cells are detected by immunofluorescence analysis. Increasing the number of viruses used for inoculation or using a different HepG2 subclone do not seem to have significant effect on the number of infected cells. In our studies, HBV virions were prepared from the supernatant culture of HepG2 cells transfected with an HBV replication-competent plasmid whereas HBV used in the published report was isolated from sera of HBV positive carriers.[14] The significantly different results between both studies may reflect the involvement of serum proteins or matrix which may be required for HBV entry. Imai *et. al.*[23] reported that a receptor for polymerized human and chimpanzee albumin on HBV particles co-occurs with hepatitis B e antigen. Plasma membranes isolated from human liver cells were also shown to bind to the spherical particles of hepatitis B surface antigen when treated with polymerized human serum albumin.[24] Moreover, the successful *in vitro* assays of HBV infection which have been reported use HBV virions derived from sera of HBV infected patients or chronic carriers for the studies.[8,13,14,25] Further investigation may be performed using serum-derived HBV for the infection to test whether or not the infection efficiency can be improved. Another possible explanation for the different results may be that the HepG2 subclones used in our studies may vary substantially from the clone used in the work

reported in the published paper. Variations in susceptibility to HBV infection have been observed with HepG2 clones or primary hepatocytes from different origins.[8,13]

Increasing the incubation periods after viral inoculation does not appear to increase the infection efficiency. However, we have noticed that many more cells die and detach at longer incubation periods. This is possibly due to a toxic effect of pH 5.5 media. As a consequence, some infected cells may be lost, thus resulting in no increase in the infection efficiency observed.

Based on our studies, another human hepatoma derived cell line, Huh-7, could not be efficiently infected with either untreated or V8-treated HBV using the published protocol. Several variable factors may be involved in this unsuccessful study, such as HBV preparation and the infection conditions, since the Huh-7 cell line was not a model culture used in the original paper.[14] Further investigations will be necessary, if Huh-7 is to be used as a culture model for the infection assay *in vitro*.

The unsuccessful infection of primary human hepatocytes with HBV possibly involves several factors. First, the primary human hepatocytes used in the study may not be healthy enough for the infection assay since cell growth may be arrested during the 48-hour-delivery at ambient temperature. Second, the detection method used for the study, *i.e.* immunochemical staining for peroxidase enzyme activity, is not appropriate as high background was detected. This may result from high level of endogenous peroxidase enzyme present in the hepatocytes or is possibly from nonspecific antibody binding. Third, as discussed above, some factors present in human sera may also be required for HBV entry; therefore, using culture-derived HBV may also have contributed to the unsuccessful infection.

In this study, however, we do detect infection of HepG2 cells by untreated HBV in acidic media, even though the infection efficiency is low, as is that of V8-treated HBV. On the basis of this finding, one may hypothesize that low pH may be required and the protease treatment of HBV may not be essential for infection of HepG2 cells. Although the

mechanism of cell entry of hepadnaviruses is still not well understood, recent evidence suggests that the process may involve endocytosis and capsid release by membrane fusion.[6,26] The pH dependence of the HBV membrane fusion environment, however, is still controversial.[6,26,27] Nonetheless, our finding seems to favor the hypothesis that acidic pH may be necessary for the membrane fusion process.[6] That HepG2 cells are refractory to HBV infection is possibly due to a defect in an acidic intracellular compartment involved in the HBV uptake pathway. Thus, incubation of cells in acidic media may trigger a cellular process to promote HBV entry. Based on this hypothesis, an HBV internalization and penetration model may be proposed. HBV may interact with a specific cellular receptor, possibly via the preS1 domain,[7,12] and the interaction may trigger a mechanism for cleavage of viral envelope proteins with presentation of the fusion domain followed by acidic pH-dependent endocytosis and membrane fusion. V8-treated HBV may bypass the earlier step of the cellular receptor interaction but still enter the cell by the same mechanism triggered by acidic pH.

Despite the discrepancy of our results compared with the published data, this study has suggested that HBVtat recombinant particles, produced from an HBV vector carrying the HIV-1 tat insert, are infectious. This finding seems to confirmed our former speculation that HBV recombinant particles produced from our HBV vectors retain infectivity since they contain the structural membrane proteins carried by the wild type HBV.[19] These results thus provide one more positive progress towards development of HBV vectors.

One might argue that the low infection efficiency observed in cells inoculated with HBV or HBVtat is possibly mediated by nonspecific receptor-independent membrane fusion. For example, human immunodeficiency virus infects receptor-negative cells at low efficiency with high concentration of virus preparation, indicating possible receptor-independent nonspecific membrane binding and entry (L.-J. Chang, personal communication). To test this speculation, further *in vitro* infection study may be performed using HBV core particles lacking the envelope protein at high concentration for the

infection. If the nonspecific internalization of HBV occurs, positive signals for HBV gene expression should also be detected in this testing experiment.

In conclusion, the *in vitro* procedure for HBV infection in HepG2 cells presented by the published report does not appear to be reproducible in our hands, thus possibly due to differences in origins of HBV and HepG2 cells used for the studies. Based on our data, protease treatment of HBV does not seem to be as important for triggering HepG2 susceptibility as does acidic pH. Further investigations are necessary. Success in developing a reproducible *in vitro* infection system for HBV in HepG2 cultures would not only be useful for determination of HBV infectivity but also provide information for understanding the early steps of the HBV replication cycle.

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CHAPTER 6

General Conclusions and Future Directions

I. Overall discussion and summary

The availability of gene therapy has provided promise for a cure for life-threatening liver diseases, such as familial hypercholesterolaemia and hemophilia B, for which an effective treatment is not presently available. The success in gene therapy for liver diseases depends mainly on the efficacy and specificity of gene delivery vehicles to target transgene expression in the liver.

The work presented in this thesis is a pioneering study on developing hepatitis B virus (HBV) as alternative liver-targeted gene delivery systems. The study was based upon the knowledge that HBV exhibits high liver specificity, both replication and gene expression. Moreover, viruses have proven to be the best natural gene transfer systems and several of them have been developed as gene therapy vectors.[1] To begin with, we had to consider several factors regarding the characteristics of HBV. First, the HBV genome is very compact and carries overlapping open reading frames (ORFs)[2] and second, almost all nucleotides of the HBV genome are included in coding sequences and are, therefore, indispensable for replication, with the principle exception of part of the tether region of the polymerase gene (P).[3,4] Third, the HBV polymerase gene product (Pol) seems to preferentially function in *cis*;[5] therefore, the HBV replication-defective construct by which the *cis* Pol protein is expressed but not functional might not be efficiently *trans*-complemented. Based on this understanding of HBV, we have developed a strategy for expressing a foreign gene by HBV by insertion of a gene into the dispensable tether region in-frame with the P gene and downstream of the preS1 promoter. Insertion in-frame with

the P gene was essential for construction of an HBV replicative vector since the Pol protein would still be expressed and allow the vector to retain its replication-competence. Using this approach, HBV replicative and nonreplicative vector systems have been developed for expression of different reporter and therapeutic genes as presented in Chapters 2, 3 and 4.

To develop expressing HBV vectors, the first and perhaps most important question to be answered was whether a foreign gene could be expressed in the HBV context. This question was addressed in Chapter 2 in which the *tat* gene of human immunodeficiency virus type 1 (HIV-1) was shown to be expressed by an HBV replicative vector with production of complete HBVtat recombinant particles. Since the *tat* gene was inserted in-frame with the P gene, this functional *tat* protein (Tat) would be in a fusion form. Although attempts to directly detect the expressed Tat were not successful, possibly due to the low quantity of the protein expression, the detection of the Tat transactivation activity and its RNAs clearly indicated the gene expression by the HBV vector. Expression of a foreign gene by an HBV vector using this approach seemed to be controlled by the preS1 promoter and/or the internal initiation mechanism of the pregenomic RNA expressed by the C promoter. To verify this hypothesis, however, further studies such as introducing mutations at the preS1 promoter and testing for expression of the *tat* gene might be useful. If mutations abolish the Tat function, the data will indicate that the functional protein is expressed by the preS1 promoter. If such a mutant retains the Tat activity, gene expression is likely to be regulated by the internal initiation mechanism. However, if such mutations result in a reduced Tat activity, the data would support the hypothesis that the regulation encompasses both the preS1 promoter and the internal initiation mechanism.

The success in the first study led to further investigations, described in Chapter 3, with two objectives. Could a gene larger than HIV-1 *tat* be expressed by an HBV replicative vector? Could HBV be constructed as a nonreplicative vector? We used a Zeocin™ resistant gene (Zeo^R) as a reporter since the size of the gene was suitable and a method for detection of the Zeo^R protein expression was available. The study of expression of Zeo^R by

an HBV replicative vector indicated that the size of a transgene to be expressed by an HBV replicative vector will be limited since it dramatically affects the replication capacity of the vector. We have shown in Chapters 2 and 3 that the HBV genome carrying a foreign gene with an additional size of about 9% or 12% of the wild type has a reduced replication capability of about 1.5% or 0.3%, respectively, of that of the wild type. A previous study showed that a DHBV recombinant genome 30% larger than the wild type failed to be packaged.[6] The nature of the functional protein product of the transgene will be another important factor to be considered since the protein expressed by an HBV replicative vector will be in the form of a fusion molecule which may cause physical constraint on the functional activity of the foreign protein and/or the polymerase protein.

As described in Chapter 3, we found that construction of HBV as a nonreplicative vector is also possible. The Pol function can be efficiently complemented *in trans*, if the expression of the *cis* Pol protein is totally eliminated. Therefore, a large foreign gene potentially can be expressed by this type of vector because part of the HBV coding sequences, such as P ORF, can be replaced.

In Chapter 4, we examined the probability of expression of a potential antitumor gene by both replicative and nonreplicative HBV vectors. We used the chicken anemia virus VP3 (CAV-VP3) or apoptin, which can induce apoptosis in tumor cells,[7,8] for the study. The apoptotic activity of this gene expressed by both HBV vectors in human hepatoblastoma cells was statistically significant compared with the negative controls. The question remains, however: is the level of expression of the gene by HBV vectors sufficient for a therapeutic purpose? Further studies to determine the gene expression *in vivo* could be pursued. One approach might be the use of mice carrying liver tumors as a study system since it has been shown that the C and preS1 regulatory elements of HBV can also function as well in mouse hepatocytes.[9] Another approach could be the use of nude mice bearing human hepatomas as a study model. This latter strategy has been employed to examine the *in vivo* expression of the apoptin gene by an adenovirus vector.[10] Using these models, the

HBV vector carrying the apoptin gene could be directly injected into the tumors and the *in vivo* antitumor gene expression could be determined by measuring alterations in the tumor morphology.

The study in Chapter 5 was performed with the important objective to determine if the *in vitro* assay for HBV infection presented by Lu and colleagues[11] could be practically applied as an *in vitro* study model for testing infectivity of HBV recombinant vector particles. In our hands, the infection efficiency obtained from using this approach was very low, possibly due to the different source of viruses and HepG2 cells used for our study. Nevertheless, the results did show that cells incubated with HBV or HBVtat particles exhibited positive signals of HBcAg and HBsAg expression, most probably due to specific receptor-mediated cellular transduction of HBV or HBVtat, respectively. If the gene expression had resulted from the nonspecific binding and entry of the viruses, highly increased multiplicity of infection would have improved the number of infected cells significantly since it should have increased the possibility of the nonspecific binding. However, it did not.

Based on the results of the study described in Chapter 5, it may be difficult to establish a reliable *in vitro* system for testing infectivity of HBV or HBV recombinant vector particles, particularly for those produced from tissue culture systems because some factors present in the host serum may be required for efficient infectivity of HBV. This hypothesis might be supported by previous studies which showed that a receptor for polymerized human and chimpanzee albumin on HBV particles co-occurs with hepatitis B e antigen[12] and that human liver cells bind to polymerized human serum albumin.[13] Furthermore, plasma membranes isolated from human liver cells were shown to bind to the spherical particles of hepatitis B surface antigen when treated with polymerized human serum albumin.[14] It should also be noted that HBV used for successful *in vitro* infection assays is derived from serum of HBV infected patients or chronic carriers.[11,15-17] Further investigations of the *in vitro* infection protocol presented by Lu *et. al.*[11] may be pursued by the addition of human

serum to the sample of HBV particles isolated from tissue culture cells to see whether or not this improves the infection efficiency. It might also be worthwhile to establish an *in vivo* animal model for testing of HBV infectivity, such as a nude mouse model harboring human hepatocytes.

In summary, there are several factors which limit further application of an HBV replicative vector for gene therapy, including the size and the nature of functional form of the transgene to be expressed and safety concerns regarding the propagation of HBV recombinant particles inside the host. Further studies must be done to verify that HBV vector particles produced from an HBV replicative vector would be attenuated and therefore, an HBV replicative vector is therapeutically applicable. In that case, this type of vector would be particularly useful for gene therapy for liver malignancies with metastasis since the therapeutic gene would also be distributed via systemic route. This type of HBV vector might be useful for therapy for liver cancer patients who are HBV positive since they may be less likely to have neutralizing immunity to the vector or to have pathogenic outcome from the replicative HBV vector infection. An HBV nonreplicative type of vector may prove to have a promising future as a gene delivery vector for gene therapy since the size of a transgene to be inserted will not be as restricted and it is potentially safe. Additionally, the transgene product expressed from this nonreplicative vector would be in its natural form.

II. Future directions for modification of HBV vectors

Expression of a transgene by both types of HBV vector would be highly liver specific because it is regulated by the preS1 and/or C promoters which exhibit strong liver specificity compared with other HBV promoter elements.[9] The expression of foreign genes by HBV vectors may be sufficient for therapeutic purposes. This will also depend on the pharmacological characteristics of an individual transgene. For example, the *tat* gene product expressed by an HBV vector exhibits the functional activity in hepatoma cells at about the same level as that expressed by the cytomegalovirus promoter. Based on these promising data, therefore, further studies and development of the HBV vector systems for liver gene transfer should be pursued.

HBV replicative vector

Further investigations of an HBV replicative vector should be done to determine the titers of recombinant vector particles produced from this type of vector, carrying different foreign genes of various sizes, to test whether the quantities are sufficient for effective gene transfer. Mutational analysis of the HBV sequence may be performed to examine the nonessential sequences which can be removed without deleterious effects on HBV replication so that the size of the transgene to be inserted could be increased. The sequence which could possibly be removed might be part of the tether region located upstream of the preS1 promoter and between the preS1 promoter and the preS1 ORF.[3,18,19]

Experiments to determine safety of use of this type of vector, *i.e.* determination of the effects of propagating HBV recombinant particles *in vivo*, must also be performed, *e.g.* in SCID mice engrafted with human liver tumor. Further studies on the possibility of attenuation or inactivation of gene expression by this vector due to the host immune

response against the HBV gene products concomitantly expressed by the vector may also be considered.

HBV nonreplicative vector

Further intensive studies will be necessary to modify an HBV nonreplicative type of vector with respect to (1) the vector construct and (2) the helper system.

Vector construct

The vector construct, which by itself is not capable of replication, should be able to express a pregenomic RNA containing the *cis*-acting elements required for both encapsidation of the pregenomic RNA and replication of the DNA genome. Therefore, several requirements must be considered prior to making modifications in the vector. First, all the essential elements required *in cis*, including the packaging signal (ε), direct repeats 1 and 2 (DR1 and DR2) and polyadenylation signal must be retained. Second, the C promoter must be included since (1) it is essential for transcription of the pregenomic RNA; (2) it appears to be the most appropriate promoter for transgene expression due to its strength and liver specificity.[9,22] The preS1 promoter may not be suitable, though it exhibits strong liver specificity, due to its weakness.[9,23] Third, the enhancer I and II elements should be included in the vector construct because they are important for liver specificity and up-regulation of the C promoter.[24-26] Retaining these important elements, the remainder of the HBV genome may be removed so that the size of the gene to be inserted can be maximized and the possibility of eliciting the host immune response to viral gene expression could be minimized. The X gene expression should be totally abolished to eliminate the potential risk of induction of hepatocellular carcinoma.[27,28] Based on this design, the P, preS1/S2/S and part of the X gene could be removed and therefore, up to about 2.5 kb of a foreign gene could be inserted.

Extensive investigations will be required to verify the efficiency and safety of a modified vector. If enhancement of the efficacy of gene expression by the HBV vector is required, a liver-specific enhancer element or an internal ribosomal entry site (IRES), which has been used to increase the efficacy of gene expression from retroviral and adenoviral vectors,[29,30] may be incorporated into the HBV vector cassette.

Helper system

The helper system can be constructed as a helper plasmid and/or a packaging cell. It, however, must provide *in trans* all the essential viral proteins required for replication of the HBV nonreplicative DNA to yield recombinant vector virions. The pregenomic RNA transcribed from this helper DNA should not be packaged or replicate, thus promoting only replication of a helper-free stock of virus particles carrying the defective vector DNA. Therefore, the ε and DR1 sequences should be deleted from the helper DNA since these elements are essential for packaging of pregenomic RNA and initiation of DNA replication.[31,32] Studies by Horwich *et. al.*[6] showed that the DHBV mutant genome containing deleted DR1 (ΔDR1) could efficiently provide *trans*-acting functions for replication of replication-defective DHBV genomes. However, the pregenomic RNA of this ΔDR1 mutant was apparently encapsidated since a small amount of the minus strand DNA was observed in cells transfected with only the ΔDR1 genome. Therefore, removal of both the ε signal and DR1 may be necessary in order to totally eliminate the possibility of encapsidation of the helper DNA. Removal of the ε element from the helper DNA would also enhance the binding and *trans*-complementation of the Pol protein to the vector DNA because its *cis*-binding site, *i.e.* the ε sequence on the helper DNA, is eliminated. The system should also be constructed such that the chance for homologous recombination to produce infectious HBV particles is eliminated or minimized. However, the potential risk of the production of HBV infectious particles by this vector should still be monitored using an efficient *in vivo* system.

This further work, along with further studies regarding gene expression and the transduction ability of the vector particles, are essential to make the HBV vector systems applicable for gene therapy.

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APPENDIX I

Reagents and Solutions

Diethylpyrocarbonate (DEPC) treated water

A 0.05% of DEPC in double-distilled water (ddH_2O) is prepared and shaken vigorously to get the DEPC into solution. The solution is incubated at 37°C at least 12 hr and autoclaved to inactivate the remaining DEPC.

Phosphate-buffered saline (PBS)

One liter of a 10X stock solution contains 80 g NaCl, 2 g KCl, 11.5 g $Na_2HPO_4.7H_2O$ and 2 g KH_2PO_4 in ddH_2O. The solution is diluted to the 1X working solution which contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$ and 1.4 mM KH_2PO_4, pH ~7.3.

Preparation of 10 %v/v protein G-sepharose

- Ten microliters of protein G-sepharose in 20% ethanol (Pharmacia Biotech, Uppsala, Sweden) are transferred to an Eppendorf tube. The supernatant is removed by centrifugation at 200 g, 4°C for 1 min. The pellet is washed 3 times in 1 ml PBS and finally resuspended in 100 µl PBS.

Trypsinization solution (ATV)

One liter of the solution contains 8 g KCl, 0.4 g NaCl, 1 g $NaHCO_3$, 0.58 g dextrose, 0.5 g trypsin (1:250) and 0.2 g disodium EDTA in ddH_2O. The solution is adjusted to pH 7.4-7.5 and filtered sterile.

Poly-L-lysine treated coverslips

Sterilization of coverslips: Coverslips are dipped in 70% ethanol and subsequently in 95% ethanol and let dry in a Lamina Flow Hood.

Coating with poly-L-lysine: Sterile coverslips are soaked in sterile poly-L-lysine (average MW 400,000) 0.1% w/v in water (Sigma Diagnostics, St. Louis, MO, USA) for 15 min and let dry in a Lamina Flow Hood in a sterile petridish.

4% paraformadehyde in PBS for cell fixing

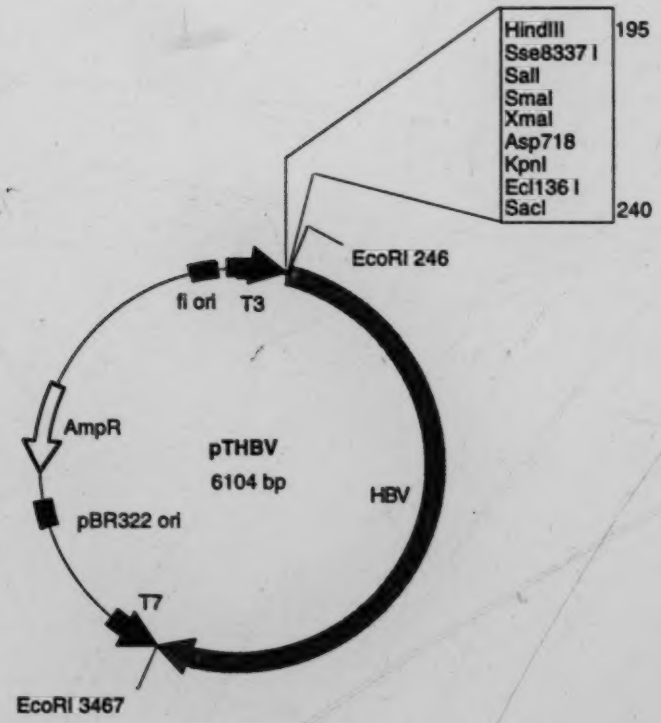
The solution is freshly prepared and used in the same day. Ten milliliters of the solution are prepared by dissolving 0.3 g paraformaldehyde in 5 ml ddH_2O with addition of one drop of 1M NaOH. To help dissolving, the solution is incubated at 50-60°C water bath in a fume hood and vortexed vigorously. After cooling down at room temperature, 5 ml of 2X PBS is added.

0.05 M Potassium phosphate buffer

The buffer is prepared by dilution of 0.1 M potassium phosphate buffer with an equal volume of ddH_2O. The 0.1 M potassium phosphate buffer is prepared as described by Ausubel F. *et. al.* (*Short Protocols in Molecular Biology 3rd Edition,* John Wiley & Sons, Inc., 1995, pp A1-46 and A1-47).

APPENDIX II

Recombinant plasmids and DNA sequences



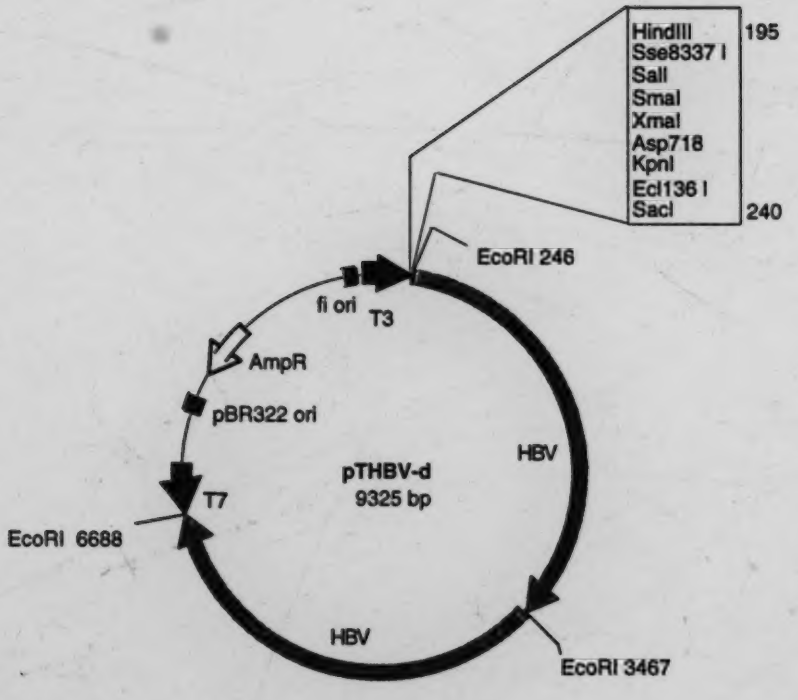
Plasmid name: pTHBV

Plasmid size: 6104 bp

Constructed by: S. Chaisomchit

Construction date: Jul. 18, 1994

Comments/ References: An EcoRI/ EcoRI fragment of HBV monomer (adw2 subtype) digested from pEC63 (constructed by L.-J. Chang) was subcloned into the EcoRI site of pT7T3 18U (Pharmacia Biotech, Uppsala, Sweden).



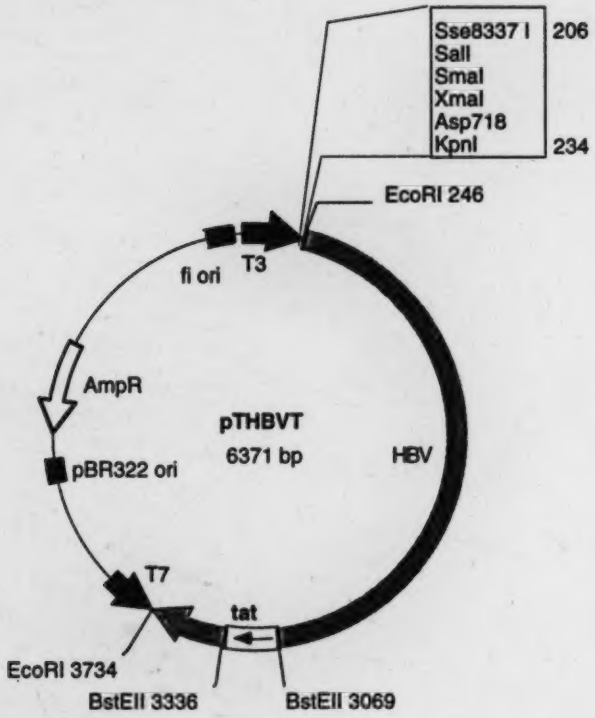
Plasmid name: pTHBV-d

Plasmid size: 9325 bp

Constructed by: S. Chaisomchit

Construction date: Jan. 12, 1995

Comments/ References: An EcoRI/ EcoRI fragment of HBV monomer was subcloned into the EcoRI site of pTHBV partially digested with EcoRI. A pTHBV-d clone carrying a head to tail tandem configuration of HBV dimers was selected by restriction mapping and DNA sequencing.



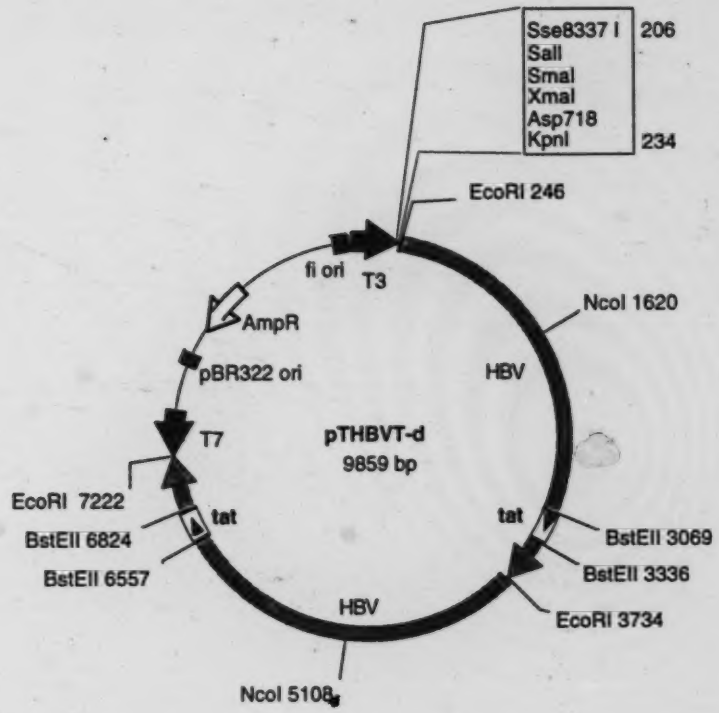
Plasmid name: pTHBVT

Plasmid size: 6371 bp

Constructed by: S. Chaisomchit

Construction date: Jul. 28, 1994

Comments/ References: The HIV-1 *tat* gene (267 bp) was subcloned from pCEP-*tat* (Robinson D. *et al*, Gene Therapy 1995; 2: 269-278) using PCR technique into the BstEII site in the tether region in-frame with the P ORF of pTHBV.



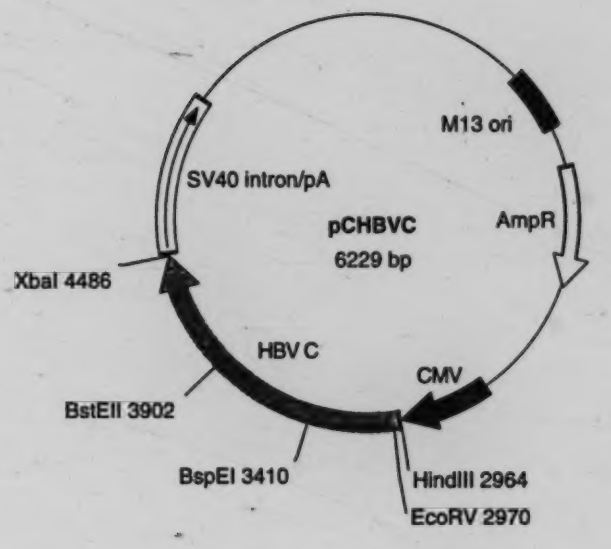
Plasmid name: pTHBVT-d

Plasmid size: 9859 bp

Constructed by: S. Chaisomchit

Construction date: May 15, 1995

Comments/References: An EcoRI/EcoRI fragment of HBVtat monomer was digested from pTHBVT and subcloned into the EcoRI site of pTHBVT partially digested with EcoRI. A pTHBVT-d clone carrying a head to tail tandem configuration of HBVtat dimers was selected by restriction mapping and DNA sequencing.



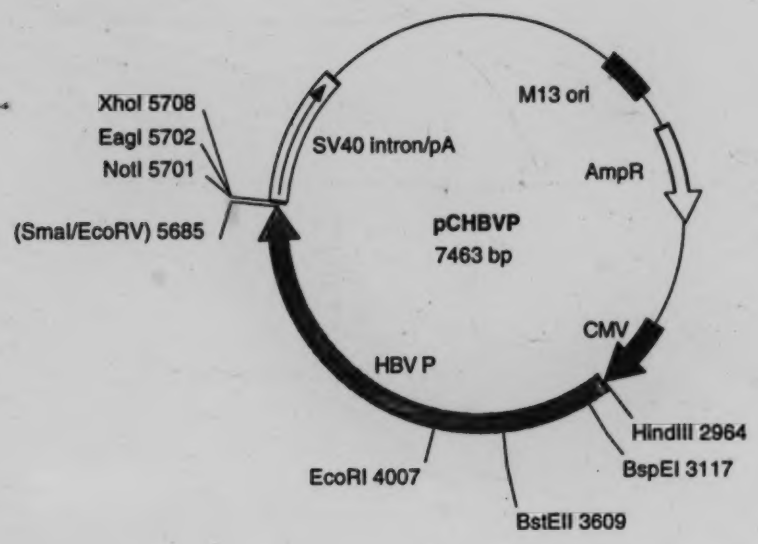
Plasmid name: pCHBVC

Plasmid size: 6229 bp

Constructed by: S. Chaisomchit

Construction date: Jun. 19, 1995

Comments/References: The entire C ORF (HindIII/XbaI fragment) of HBV adw991 subtype (Seifer M. *et al*, Virology 1990; 179: 1388-1391) was subcloned from pBHBVC-1 (constructed by Z. Li) into the HindIII/XbaI sites of pcDNA I Amp (Invitrogen, San Diego, CA, USA). Expression of the C gene was controlled by the CMV promoter. The initiation codon for translation of the C gene was located at the 2982 nt.



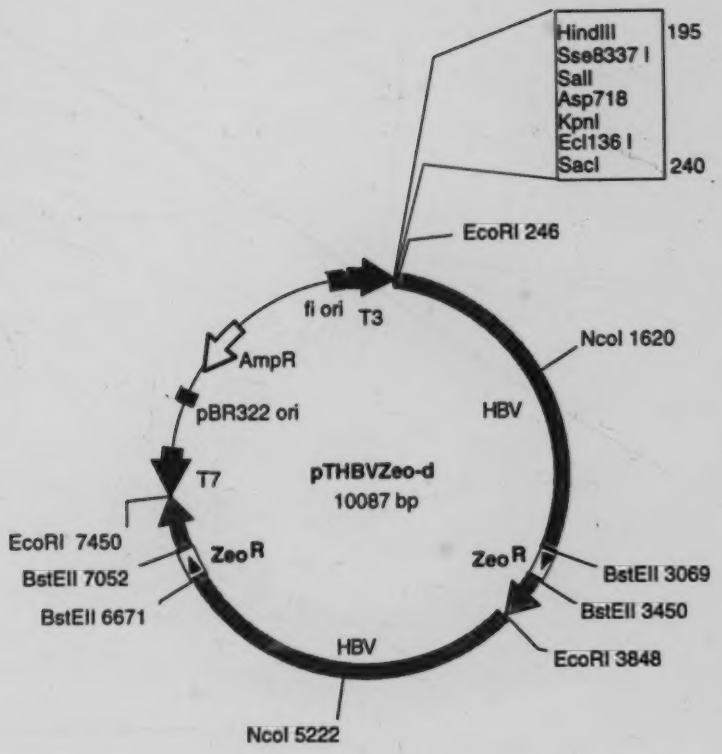
Plasmid name: pCHBVP

Plasmid size: 7463 bp

Constructed by: S. Chaisomchit

Construction date: Jun. 19, 1995

Comments/References: The entire P ORF (HindIII/SmaI fragment) of HBV adw 991 subtype (Seifer M. *et al*, Virology 1990; 179: 1388-1391) was subcloned from pTZKSVHBVP991 (constructed by Z. Li) into the unique HindIII/EcoRV sites of pcDNA I Amp. Expression of the P gene was controlled by the CMV promoter. The initiation codon for translationof the P gene was located at the 3095 nt.



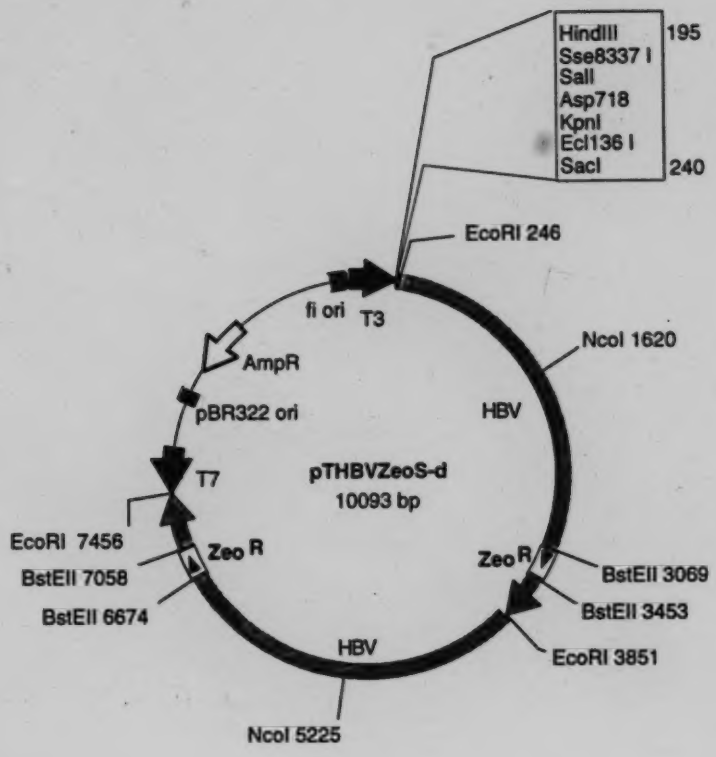
Plasmid name: pTHBVZeo-d

Plasmid size: 10087 bp

Constructed by: S. Chaisomchit

Construction date: Oct 29, 1996

Comments/References: An EcoRV EcoRI fragment of HBVZeo monomer was digested from pTHBVZeo [constructed by subcloning the Zeo^R gene from pcDNA3.1/ Zeo (Invitrogen, San Diego, CA, USA) into the BstEII site and in-frame with the HBV P ORF of pTHBV] and subcloned into the EcoRI site of pTHBVZeo partially digested with EcoRI. A pTHBVZeo-d clone carrying a head to tail tandem configuration of HBVZeo dimers was selected by restriction mapping and DNA sequencing.



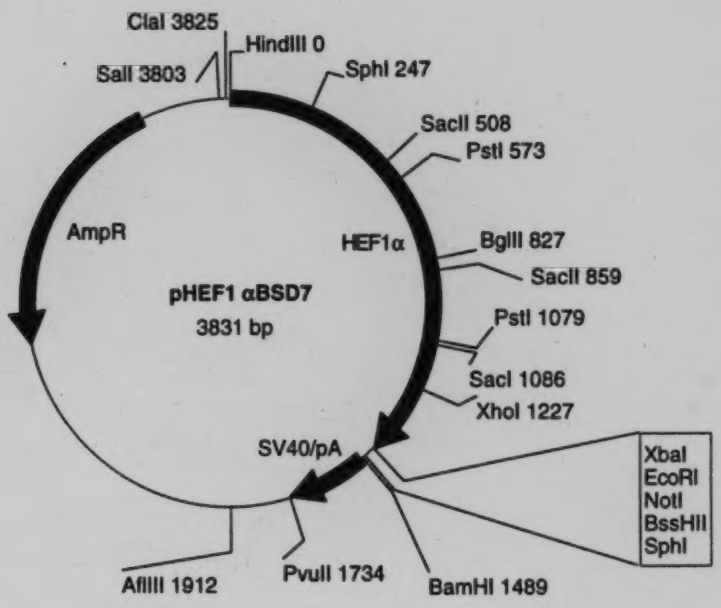
Plasmid name: pTHBVZeoS-d

Plasmid size: 10093 bp

Constructed by: S. Chaisomchit

Construction date: Mar. 3, 1997

Comments/References: An EcoRI/ EcoRI fragment of HBVZeoS monomer was digested from pTHBVZeoS (constructed by subcloning the Zeo^R gene with a stop codon from pcDNA3.1/ Zeo into the BstEII site and in-frame with the HBV P ORF of pTHBV) and subcloned into the EcoRI site of pTHBVZeoS partially digestion with EcoRI. A pTHBVZeoS-d clone carrying a head to tail tandem configuration of HBVZeoS dimers was selected by restriction mapping and DNA sequencing.



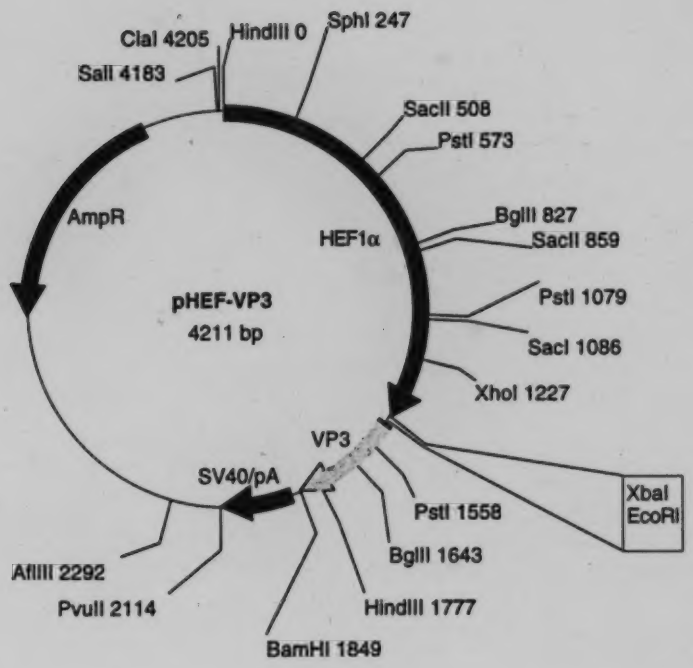
Plasmid name: pHEF1αBSD7

Plasmid size: 3831 bp

Constructed by: D. Daney

Construction date: 1992

Comments/References: Human elonagtion factor 1 alpha enhancer (bases 125-1567); Uetsuki T. *et al,* J. Biol. Chem. 1989; 264: 5791 and Mizushima S. and Nagata S., Nucleic Acids Res. 1990; 18: 5322. Transcription starts at 455 nt. Intron starts from 488 nt to 1431 nt, 52 bp 5' UT through XbaI site in mature mRNA.



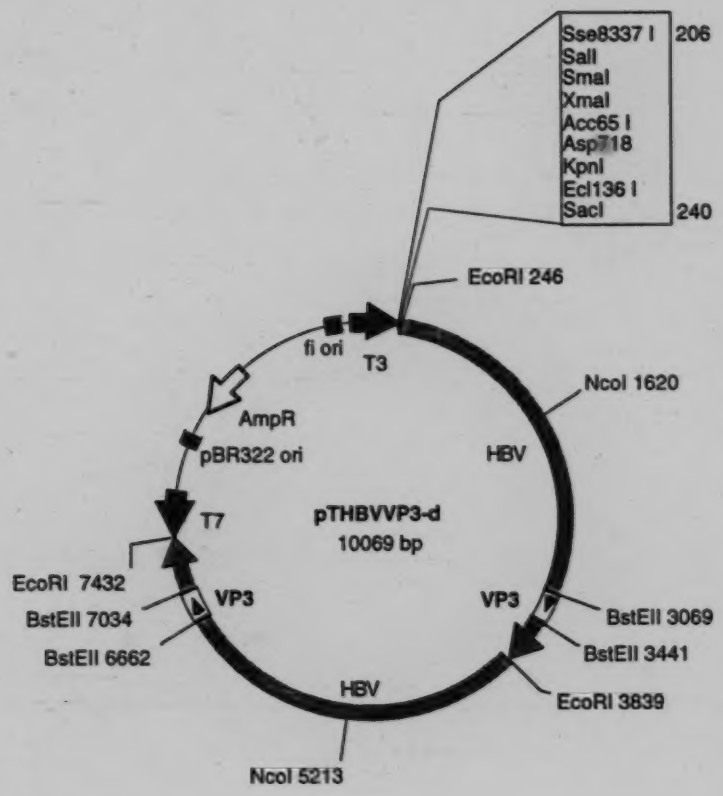
Plasmid name: pHEF-VP3

Plasmid size: 4211 bp

Constructed by: Y. Cui

Construction date: Feb. 6, 1997

Comments/ References: The CAV-VP3 was amplified from pCAA-5 (Meehan B. M. *et al*, Arch. Virol. 1992; 124: 301-319) with primers VP3-3'-BamHI and VP3-5'-EcoRI using Pfu polymerase. BamHI and EcoRI digested fragment was cloned into vector pHEF1αBSD7.



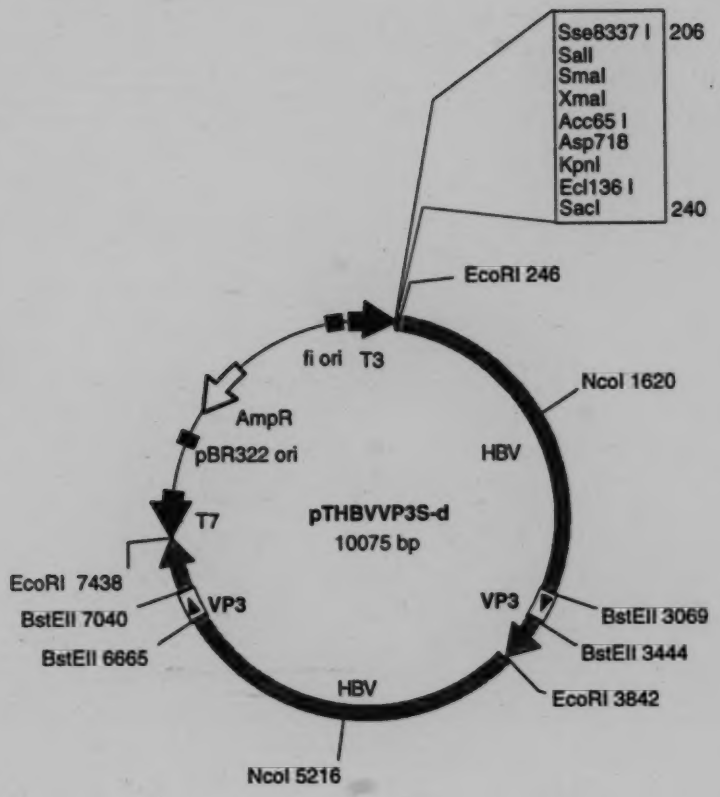
Plasmid name: pTHBVVP3-d

Plasmid size: 10069 bp

Constructed by: S. Chaisomchit

Construction date: Nov. 5, 1997

Comments/References: An EcoRI/EcoRI fragment of HBVVP3 monomer was digested from pTHBVVP3 (constructed by subcloning the CAV-VP3 gene from pHEF-VP3 into the BstEII site and in-frame with the HBV P ORF of pTHBV) and subcloned into the EcoRI site of pTHBVVP3 partially digested with EcoRI. A pTHBVVP3-d clone carrying a head to tail tandem configuration of HBVVP3 dimers was selected by restriction mapping and DNA sequencing.



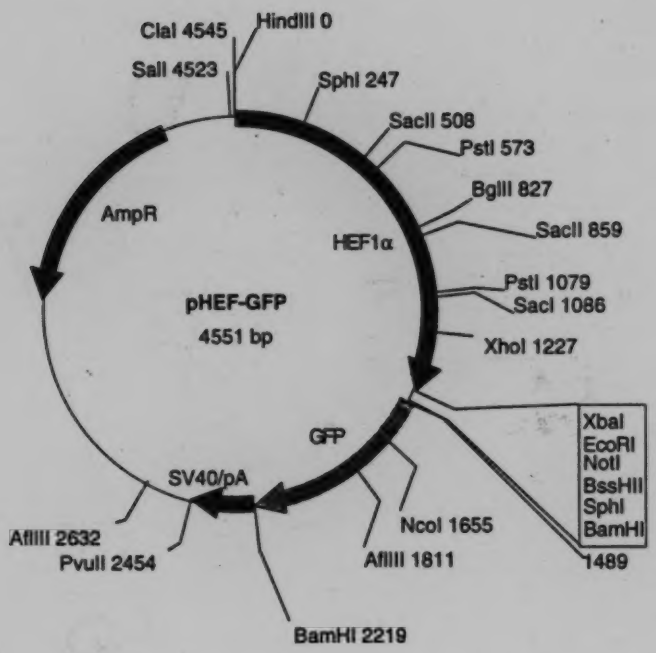
Plasmid name: pTHBVVP3S-d

Plasmid size: 10075 bp

Constructed by: S. Chaisomchit

Construction date: Oct. 6, 1997

Comments/References: An EcoRI/ EcoRI fragment of HBVVP3S monomer was digested from pTHBVVP3S (constructed by subcloning the CAV-VP3 gene with a stop codon from pHEF-VP3 into the BstEII site and in-frame with the HBV P ORF of pTHBV) and subcloned into the EcoRI site of pTHBVVP3S partially digested with EcoRI. A pTHBVVP3S-d clone carrying a head to tail tandem configuration of HBVVP3S dimers was selected by restriction mapping and DNA sequencing.



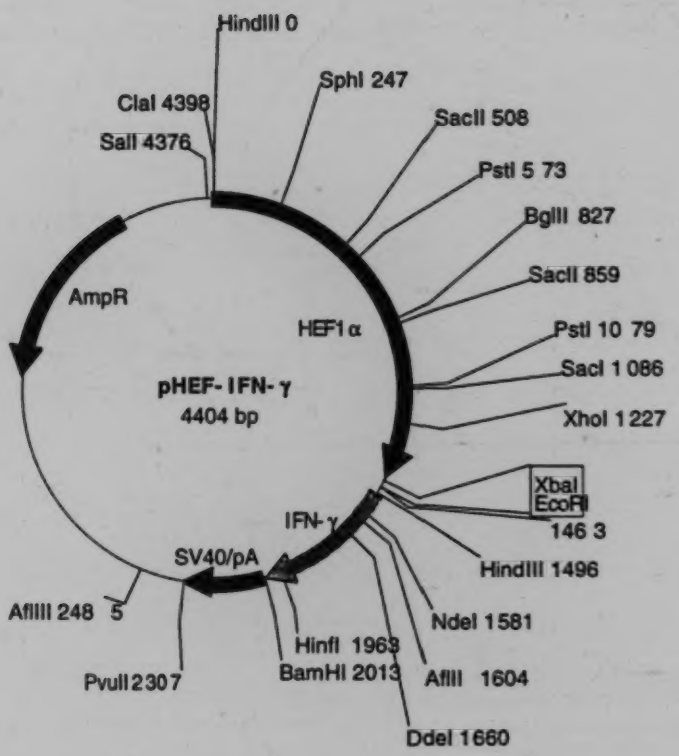
Plasmid name: pHEF-GFP

Plasmid size: 4551 bp

Constructed by: L.-J. Chang

Construction date: Oct. 2, 19 95

Comments / References: GFP was amplified by PCR and cloned into pBSIIKS to get pBS-GFP#2. The BamHI fragment (~730 bp) containing the GFP ORF was cut and further subcloned into the BamHI site of pHEF1αBSD7.



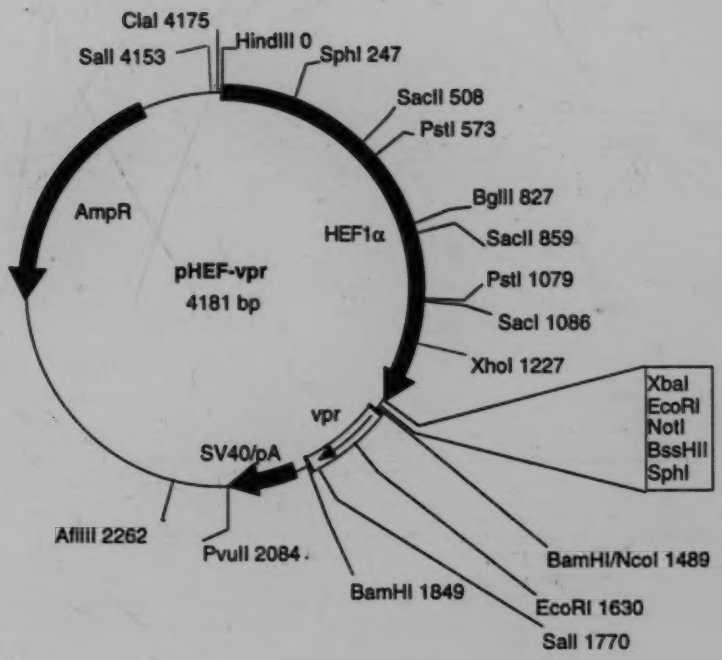
Plasmid name: pHEF-IFN-γ

Plasmid size: 4404 bp

Constructed by: Y. Cui

Construction date: Dec. 8, 1996

Comments/References: EcoRI and BamHI digested fragments from pHEF1αBSD7 and pLSNIFN-γ were religated.



Plasmid name: pHEF-vpr

Plasmid size: 4181 bp

Constructed by: L.-J. Chang

Construction date: Oct. 7, 1995

Comments/References: pHEF1αBSD7 cut with BamHI and CIP treated, was ligated with pLSN-vpr fragment cut with BamHI (~360 bp).

HIV-1 *tat* gene

ATGGAGCCAGTAGATCCTAATCTAGAGCCCTGGAAGCATCCAGGAAGTCAGCC
TAAAACTGCTTGTACCAATTGCTATTGTAAAAAGTGTTGCTTTCATTGCCAAGTT
TGTTTCATGACAAAAGCCTTAGGCATCTCCTATGGCAGGAAGAAGCGGAGACA
GCGACGAAGAGCTCATCAGAACAGTCAGACTCATCAAGCTTCTCTATCAAAGCA
ACCCACCTCCCAATCCCGAGGGGACCCGACAGGGCCCACGGAATAG

Zeocin™ resistant gene (Zeo^R)

ATGGCCAAGTTGACCAGTGCCGTTCCGGTGCTCACCGCGCGCGACGTCGCCG
GAGCGGTCGAGTTCTGGACCGACCGGCTCGGGTTCTCCCGGGACTTCGTGGA
GGACGACTTCGCCGGTGTGGTCCGGGACGACGTGACCCTGTTCATCAGCGCG
GTCCAGGACCAGGTGGTGCCGGACAACACCCTGGCCTGGGTGTGGGTGCGCG
GCCTGGACGAGCTGTACGCCGAGTGGTCGGAGGTCGTGTCCACGAACTTCCG
GGACGCCTCCGGGCCGGCCATGACCGAGATCGGCGAGCAGCCGTGGGGGCG
GGAGTTCGCCCTGCGCGACCCGGCCGGCAACTGCGTGCACTTCGTGGCCGAG
GAGCAGGACTGA

CAV-VP3 gene

ATGAACGCTCTCCAAGAAGATACTCCACCCGGACCATCAACGGTGTTCAGGCC
ACCAACAAGTTCACGGCCGTTGGAAACCCCTCACTGCAGAGAGATCCGGATTG
GTATCGCTGGAATTACAATCACTCTATCGCTGTGTGGCTGCGCGAATGCTCGCG
CTCCCACGCTAAGATCTGCAACTGCGGACAATTCAGAAAGCACTGGTTCCAAG
AATGTGCCGGACTTGAGGACCGATCAACCCAAGCCTCCCTCGAAGAAGCGATC
CTGCGACCCCTCCGAGTACAGGGTAAGCGAGCTAAAAGAAAGCTTGATTACCA
CTACTCCCAGCCGACCCCGAACCGCAAGAAGGTGTATAAGACTGTAA